【岡村智教】

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Kuwabara K, Okamura T, et al.	The relationship between non-HDL-C and LDL-C in the general population: the KOBE Study and Tsuruoka Metabolomic Cohort Study.	J Atheroscler Thromb	in press	in press	2016

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【宮本恵宏】

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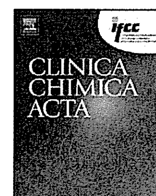

HDL cholesterol performance using an ultracentrifugation reference measurement procedure and the designated comparison method ☆

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ABSTRACT

Background: Accurate high-density lipoprotein cholesterol (HDL-C) measurements are important for management of cardiovascular diseases. The US Centers for Disease Control and Prevention (CDC) and Cholesterol Reference Method Laboratory Network (CRMLN) perform ultracentrifugation (UC) reference measurement procedure (RMP) to value assign HDL-C. Japanese CRMLN laboratory (Osaka) concurrently runs UC procedure and the designated comparison method (DCM). Osaka performance of UC and DCM was examined and compared with CDC RMP.

Methods: CDC RMP involved UC, heparin-$MnCl_2$ precipitation, and cholesterol analysis. CRMLN DCM for samples containing <200 mg/dl triglycerides involved 50-kDa dextran sulfate-$MgCl_2$ precipitation and cholesterol determination.

Results: HDL-C regression equations obtained with CDC (x) and Osaka (y) were $y = 0.992x + 0.542$ ($R^2 = 0.996$) for Osaka UC and $y = 1.004x - 0.181$ ($R^2 = 0.998$) for DCM. Pass rates within ±1 mg/dl of the CDC target value were 91.9 and 92.1% for Osaka UC and DCM, respectively. Biases at 40 mg/dl HDL-C were +0.22 and −0.02 mg/dl for Osaka UC and DCM, respectively.

Conclusions: Osaka UC and DCM were highly accurate, precise, and stable for many years, assisting manufacturers to calibrate products for clinical laboratories to accurately measure HDL-C for patients, calculate non-HDL-C, and estimate low-density lipoprotein cholesterol with the Friedewald equation.

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1. Introduction

A low high-density lipoprotein cholesterol (HDL-C) level is a strong biomarker for predicting the risk of cardiovascular diseases (CVD), as demonstrated by several epidemiological studies and clinical trials [1–3]. The US National Cholesterol Education Program (NCEP) estimated that each 1% increase in HDL-C may be associated with a 2–4% decrease in the risk of coronary heart disease (CHD), and clinical trials on low-density lipoprotein-lowering therapies have shown that concomitant increases in HDL-C confer an additional independent reduction in the risk of CHD [4]. HDL-C together with low-density lipoprotein cholesterol (LDL-C), total cholesterol, and triglycerides form a lipid panel that is measured in routine patient care to determine and monitor the risk of a patient developing CVD.

Accurate and reproducible HDL-C measurements are of particular importance for correctly and consistently classifying individuals at risk of CVD, as outlined in the clinical guidelines for the subsequent

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diagnosis, treatment, and prevention of patients [5–7]. Furthermore, the US NCEP reported [4] that the accuracy of HDL-C was particularly important because (a) the inverse association of HDL-C with the risk of CHD is expressed over a relatively narrow concentration range, (b) the medical decision cut-off point (40 mg/dl) for an increased risk of CHD is at the lower end of the HDL-C concentration range, at which small errors can have a strong impact on patient classification, and (c) the calculation of non-HDL-C [8,9] or LDL-C using the Friedewald equation [10]. Inaccurate HDL-C measurements also lead to errors in the estimation of LDL-C.

Previous studies recommended that the US Centers for Disease Control and Prevention (CDC) reference measurement procedure (RMP) should be used to achieve accurate HDL-C measurements. CDC RMP is a three-step procedure [11,12]: (1) ultracentrifugation (UC) at d = 1.006 kg/l to remove triglyceride-rich lipoproteins; (2) precipitation of apo B-containing lipoproteins from the ultracentrifugal infranatant with heparin-$MnCl_2$; (3) measurement of cholesterol in the heparin-$MnCl_2$ supernatant using the CDC reference method for cholesterol [13]. However, ultracentrifugal measurements of HDL-C have low sample throughput and require equipment that is not commonly available in routine clinical laboratories. Therefore, the Cholesterol Reference Method Laboratory Network (CRMLN) sought to implement a designated comparison method (DCM) [14–16] with the objective of better assisting reagent manufacturers in the calibration of their products so that clinical laboratories could more accurately measure HDL-C for patients, calculate non-HDL-C, and estimate LDL-C with the Friedewald equation.

The CDC UC method has been accepted as the most reliable RMP for HDL-C and the CRMLN DCM is an accurate, robust, transferable and practical method for clinical laboratories and manufacturers. As part of the CRMLN activities, the National Cerebral and Cardiovascular Center at Osaka, Japan has implemented and maintained 1) the UC method, which is same as CDC RMP, for 17 years since May 1997 and 2) DCM for 20 years since April 1994. We measured the performance of both HDL-C reference methods in terms of accuracy and reproducibility after many years using comparisons with CDC RMP.

2. Materials and methods

2.1. Materials

All standardization pools for HDL-C were prepared according to the Clinical Laboratory Standards Institute document C37-A (Preparation and Validation of Commutable Frozen Human Serum Pools as Secondary Reference Materials for Cholesterol Measurement Procedures; Approved Guideline), which implied that no preservatives or no additives were added. All survey pools were blinded to the CRMLN laboratories. They were shipped frozen from CDC and stored at −70 °C before analysis.

HDL-C assays were conducted in the Osaka Medical Center for Cancer and Cardiovascular Diseases between July 1997 and June 2001, in the Osaka Medical Center for Health Science and Promotion between July 2001 and March 2012, and in the National Cerebral and Cardiovascular Center at Osaka continuously from April 2012 (all laboratories were referred to as the 'Osaka' laboratory).

2.2. Methods

2.2.1. CDC reference measurement procedure for ultracentrifugation

The first step of CDC RMP employed preparative ultracentrifugation (Beckman Coulter, Optima L-70 K and/or Optima XE-90) to remove apo B-containing lipoproteins [11,12]. The methods at CDC and Osaka used 5.00 ml of serum at a density of d = 1.006 kg/l (0.195 mol/l NaCL solution) and a 50.4Ti rotor (Beckman Coulter). UC at CDC was carried out for 16.2 h at 120,000 ×g and 18 °C, and at Osaka for 18.5 h at 105,000 ×g and 18 °C. After UC, the top fraction (d < 1.006 kg/l) was removed using tube slicer and the bottom fraction (d > 1.006 kg/l) was quantitatively transferred to a 5.00 ml volumetric flask adjusting with 0.15 mol/l NaCl solution [14–16]. In the second step, 1.00 ml aliquots of the bottom fraction were precipitated with 40 μL heparin (sodium injection, 5000 USP units/ml, Baxter Healthcare Corporation) and 50 μL manganese reagents ($MnCl_2$ solution, 1.00 M ±0.01 M, SIGMA) [17]. The precipitate was removed for 30 min at 1500 ×g and 4 °C [18–20]. In the third step, HDL-C was determined in the supernatant in duplicate measurements by the Abell–Kendall reference method for cholesterol [13]. The recovered cholesterol value was multiplied by 1.09 to account for the dilution introduced by the addition of the precipitation reagent. Four replicates from each sample were used in comparisons of assay performance.

2.2.2. CRMLN designated comparison method

DCM is a precipitation-based designated comparison method using 50-kDa dextran sulfate (DS)-$MgCl_2$ as the reagent. DS (stored at 2 to 8 °C. Kept tightly capped in a desiccator in a refrigerator after opening) was obtained from Warnick & Co. and was a special lot (lot#: 162176) for CRMLN use only. All CRMLN laboratories used the same DS lot to minimize potential lot-to-lot variations. $MgCl_2 \cdot 6H_2O$ (this reagent was highly hygroscopic and had to be dried. A larger amount than was needed was placed in a beaker and dried in an oven at 37 °C for at least one hour) and sodium azide (NaN_3) were obtained from Wako Pure Chemistries Inc. in Japan. The stock solution of DS contained 2.0 g/dl DS including 50 mg/dl NaN_3, while that of $MgCl_2$ contained 14.22 g/dl including 50 mg/dl NaN_3. The working reagent was prepared by mixing equal volumes. The working solution was stored at 2 and 8 °C [15,16]. Osaka laboratory previously confirmed that it was stable for 3 years.

In the first step, the samples and working reagent were equilibrated to room temperature and mixed at a ratio of 1.00 ml specimen and 0.10 ml working reagent. The samples for DCM required normotriglyceridemic sera including <200 mg/dl in triglycerides because of its limited sedimentation efficiency [16,17]. The samples were then incubated at room temperature for 10–30 min and centrifuged for 30 min at 4 °C at 1500 ×g. In the second step, clear supernatants were analyzed using the reference method for cholesterol [13]. The recovered HDL-cholesterol value in the DCM was multiplied by 1.1. HDL-C was assayed in the supernatant in duplicate measurements. Four replicates from one aliquot were used in comparisons of assay performance.

2.3. Performance criteria for HDL-C applied to CRMLN laboratories

The performance criteria for HDL-C applied to the CRMLN lipid reference laboratories are summarized in Table 1. Imprecision is evaluated not in coefficient variation (CV), but in standard deviation (SD, unit: mg/dl), and accuracy is evaluated in bias (mg/dl) from CDC reference value.

2.4. Statistical analysis

We used protocol EP9-A from the Clinical and Laboratory Standards Institute for bias estimation [21] and the STATA12 analysis program for all other calculations.

Table 1
Performance criteria applied to CRMLN lipid reference laboratory using UC method and DCM for HDL-C.

Lipid	Imprecision criterion	Accuracy criterion
HDL-C	Standard deviation ≤1 mg/dl	Bias ≤1 mg/dl

CRMLN: cholesterol reference method laboratory network. UC: ultracentrifugation. DCM: designated comparison method. HDL-C: high-density lipoprotein cholesterol.

3. Results

3.1. Accuracy

In the UC procedure at Osaka, the pooled serum with 160 different concentrations (lots) for HDL-C were analyzed among 626 survey samples with 154 survey runs, in which each survey run consisted of 3 to 5 different pools. They were analyzed for 17 years between May 1997 and January 2014. The concentration ranges were 26.9–78.9 mg/dl. In the scatter plots of bias (unit: mg/dl) between Osaka (y) and CDC (x), $y = -0.008x + 0.540$ ($R^2 = 0.017$). The p-values and 95% confidence interval (CI) of the slopes and intercepts were 0.001 and (−0.013, −0.003), and <0.001 and (0.296, 0.784), respectively (Table 2). The Osaka laboratory met acceptable accuracy goals for 91.9% (575 of 626 samples) within ±1 mg/dl of the CDC reference values (Fig. 1A). Biases between the target values of CDC and the measurements of Osaka at two medical decision points of 40 and 60 mg/dl were 0.22 and 0.06 mg/dl, respectively, both of which were slightly on the positive side. Although the bias and SD scattering of DCM appeared to be slightly better than that of CDC RMP, no significant differences (p-value: 0.05) were observed in the accuracy or precision of the 2 procedures.

In the DCM at Osaka, the pooled serum with 163 different concentrations (lots) for HDL-C were analyzed among 570 survey samples with 147 survey runs, in which each survey run consisted of 3 to 4 different pools. They were analyzed for 20 years between April 1994 and January 2014. The concentration ranges were 20.8–86.0 mg/dl. In the scatter plots of bias (unit: mg/dl) between Osaka (y) and CDC (x), $y = 0.004x - 0.181$ ($R^2 = 0.006$). The p-values and 95% CI of the slopes and intercepts were 0.065 and (−0.0002, 0.007), and 0.062 and (−0.370, 0.009), respectively (Table 2). The Osaka laboratory met acceptable accuracy goals for 92.1% (525 of 570 samples) within ±1 mg/dl of the CDC reference values (Fig. 1B). Biases between the target values of CDC and measurements of Osaka at two medical decision points of 40 and 60 mg/dl were −0.02 and +0.06 mg/dl, respectively, both of which were slightly biased.

3.2. Precision

In the scatter plots of SD between Osaka (y) and CDC (x), y (SD, mg/dl) = 0.002x (CDC reference value) + 0.270 [n: 626, $R^2 = 0.006$]. The p-value and 95% CI for the slope were 0.056 and (−0.00005, 0.0036), respectively. The p-value and 95% CI for the intercept were <0.001 and (0.179, 0.360), respectively (Table 2). The Osaka laboratory met acceptable precision goals for 97.9% (613 of 626 samples) within ±1 mg/dl. The maximum SD at Osaka UC was 2.3 mg/dl (Fig. 2C).

In the scatter plots of SD between Osaka (y) and CDC (x), y (SD, mg/dl) = 0.001x (CDC reference value) + 0.218 [n: 570, $R^2 = 0.005$].

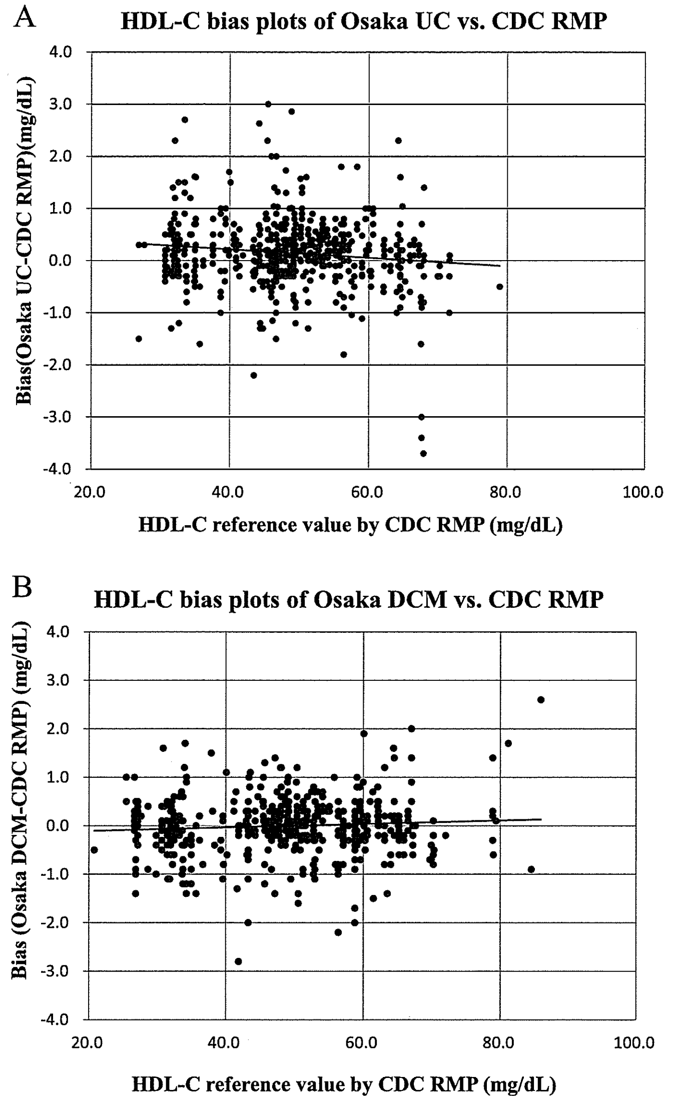

Fig. 1. A: HDL-C bias plots of Osaka UC vs. CDC RMP. The y-axis indicates the bias (mg/dl) of Osaka UC compared to the CDC reference value and the x-axis indicates the CDC RMP HDL-C reference value. CDC: The US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol. UC: Ultracentrifugation. B: HDL-C bias plots of Osaka DCM vs. CDC RMP. The y-axis indicates the bias (mg/dl) of the Osaka DCM compared to the CDC reference value and the x-axis indicates the CDC RMP HDL-C reference value. CDC: The US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol. DCM: Designated comparison method.

Table 2
Regression analysis of the bias between Osaka (y) and CDC (x) and imprecision for HDL-C over time (unit: mg/dl).

Parameter	HDL-C method	Number of samples	Slope (95%CI)	Intercept (95%CI)	R^2	Time period
Accuracy	UC	626	−0.008 (−0.013, −0.003) p = 0.001	0.540 (0.296, 0.784) p < 0.001	0.017	May 1997 to January 2014 (17 years)
	DCM	570	0.004 (−0.0002, 0.07) p = NS	−0.181 (−0.370, 0.009) p = NS	0.006	April 1994 to January 2014 (20 years)
Precision	UC	626	0.002 (−0.00005, 0.0036) p = NS	0.270 (0.179, 0.360) p < 0.001	0.006	May 1997 to January 2014 (17 years)
	DCM	570	0.001 (−0.0001, 0.002) p = NS	0.218 (0.162, 0.275) p < 0.001	0.005	April 1994 to January 2014 (20 years)

UC: ultracentrifugation. DCM: designated comparison method.

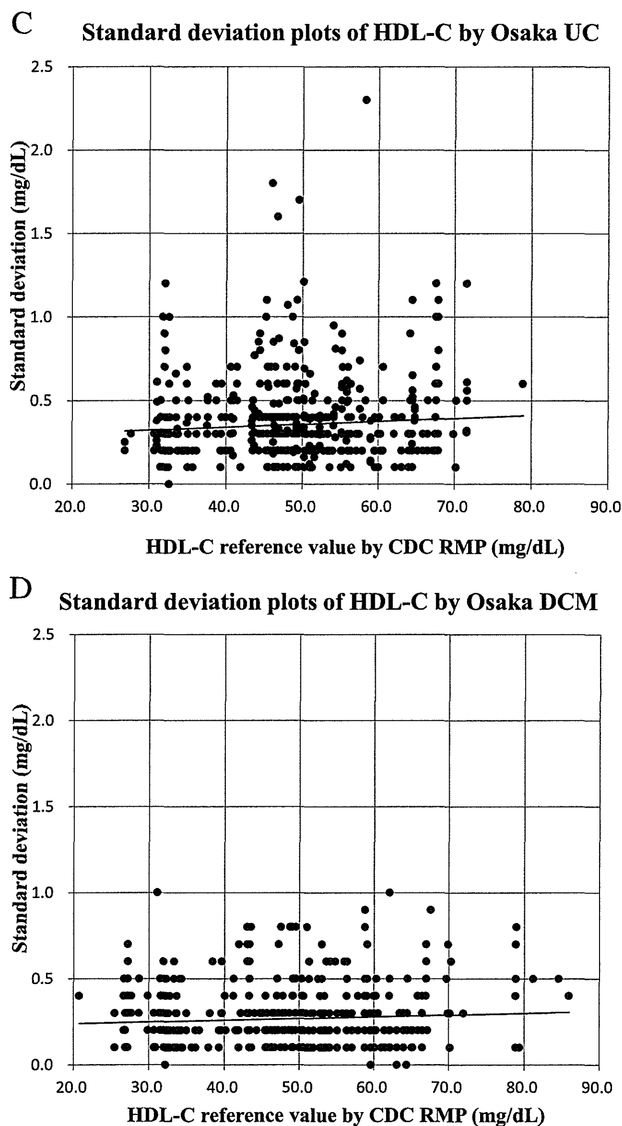

Fig. 2. C: Standard deviation plots of HDL-C by Osaka UC. The y-axis indicates the SD (mg/dl) of the Osaka UC method compared to the CDC reference value and the x-axis indicates the CDC RMP HDL-C reference value. CDC: The US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol. D: Standard deviation plots of HDL-C by Osaka DCM. The y-axis indicates the SD (mg/dl) of the Osaka DCM compared to the CDC reference value and the x-axis indicates the CDC RMP HDL-C reference value. CDC: The US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol. DCM: Designated comparison method.

The p-value and 95% CI for the slope were 0.083 and (−0.0001, 0.002), respectively. The p-value and 95% CI for the intercept were <0.001 and (0.162, 0.275), respectively (Table 2). The Osaka laboratory met acceptable precision goals for 100.0% (all 570 samples) within ±1 mg/dl. The maximum SD at Osaka DCM was 1.0 mg/dl (Fig. 2D).

3.3. Long-term bias (mg/dl) plots by the UC method and DCM at Osaka

Fig. 3E shows the bias (mg/dl) plots of Osaka UC HDL-C vs. CDC RMP at each run for 17 years. The minimum value of the bias was −3.7 mg/dl while the maximum value was 3.0 mg/dl. The x-axis indicated the survey run number between May 1997 and January 2014 with 154 runs and the y-axis indicated the bias (mg/dl) of Osaka UC HDL-C vs. CDC RMP. The acceptable criteria for the accuracy of HDL-C were within ±1.0 mg/dl of the target value of CDC. Each survey run consisted of 3 to 5 CDC pools for the HDL-C analysis.

Fig. 3F shows the bias (mg/dl) plots of Osaka DCM HDL-C vs. CDC RMP at each run for 20 years. The minimum value of the bias was −2.8 mg/dl while the maximum value was 2.6 mg/dl. The x-axis indicated the survey run number between April 1994 and January 2014 with 147 runs and the y-axis indicated the bias (mg/dl) of Osaka DCM HDL-C vs. CDC RMP. The acceptable criteria for the accuracy of HDL-C were within ±1.0 mg/dl of the target value of CDC. Each survey run consisted of 3 to 4 CDC pools for the HDL-C analysis.

4. Discussion

Previous epidemiological studies and clinical trials were based on the results of large scale population studies using the UC method for HDL-C, which were, in turn, based on the heparin-$MnCl_2$ precipitation method. However, an inherent problem with this precipitation method is the inability to sediment all the centrifuged lipoproteins [18,19], which mainly affects triglyceride-rich lipoproteins included in turbid or milky diseased specimens. Therefore, the UC procedure merits the elimination of interference [20,22].

High-density lipoprotein (HDL) represents a mixture of heterogeneous macromolecules and physicochemical particles. No primary certified standards or measurement procedures are currently available for HDL-C in order to establish the metrological traceability of HDL-C measurements to SI. However, UC-based CDC RMP has been the reference method of HDL-C measurements for practical use. DCM was established to better meet needs related to faster sample turnaround and higher throughput [16]. Both methods are now used to assure the accuracy of testing performed in patient care and research. However, it is important to understand the limitations of the DCM, especially with samples containing high levels of triglycerides. Therefore, it will be necessary and important to maintain the UC-based reference method and its standardization when encountering diseased and complicated samples.

Iso et al. in the Circulatory Risk in Communities Study (CIRCS) have conducted epidemiological studies on the prevention of and reductions in cerebral strokes and heart diseases among Japanese individuals for over 50 years [2,9]. During this time, we have experienced various changes for HDL-C in assay principles from the old precipitation methods to new homogeneous methods, in instruments from manual operation to automatic analyzers, in reagents from strong acids to mild enzymes, and in calibrators from cholesterol standards in alcohol to serum-based materials. All these changes have influenced the precision and accuracy of HDL-C measurements. Therefore, it is of utmost importance to ensure reference methods providing an accuracy basis for clinical measurements remain consistent and stable over time. This is achieved by maintaining a network of reference laboratories. In the present study, we assessed the measurement performance and limits of the UC and DCM methods for HDL-C at Osaka.

The homogeneous HDL-C reagents now widely adopted have several advantages: they are fully automated on various analytical instruments, have good precision, triglycerides do not need to be measured, and non-fasting samples may potentially be used. However, Miller et al. [23] and Miida et al. [24] reported some limitations when comparing these assays against the UC-RMP. Deventer et al. found that non-HDL cholesterol showed improved accuracy for cardiovascular risk score classification over that of direct or calculated LDL cholesterol in a dyslipidemic population [25]. Non-HDL-C is calculated as [total cholesterol − HDL-C]. Therefore, accurate HDL-C will be a key factor in obtaining accurate non-HDL-C values. Since non-HDL-C was previously reported to be superior to LDL-C in predicting the risk of CVD risk [8,9], it will be recommended as a primary screening test in the future by Japanese authorities.

Recent discovery that serum/plasma HDL-C markedly and selectively increased by up to 15% over the past 20 years among Japanese individuals [26] raised concerns regarding consistencies in HDL-C measurements in Japan. According to the Japanese National Health and Nutritional Survey, the average HDL-C levels of males and females

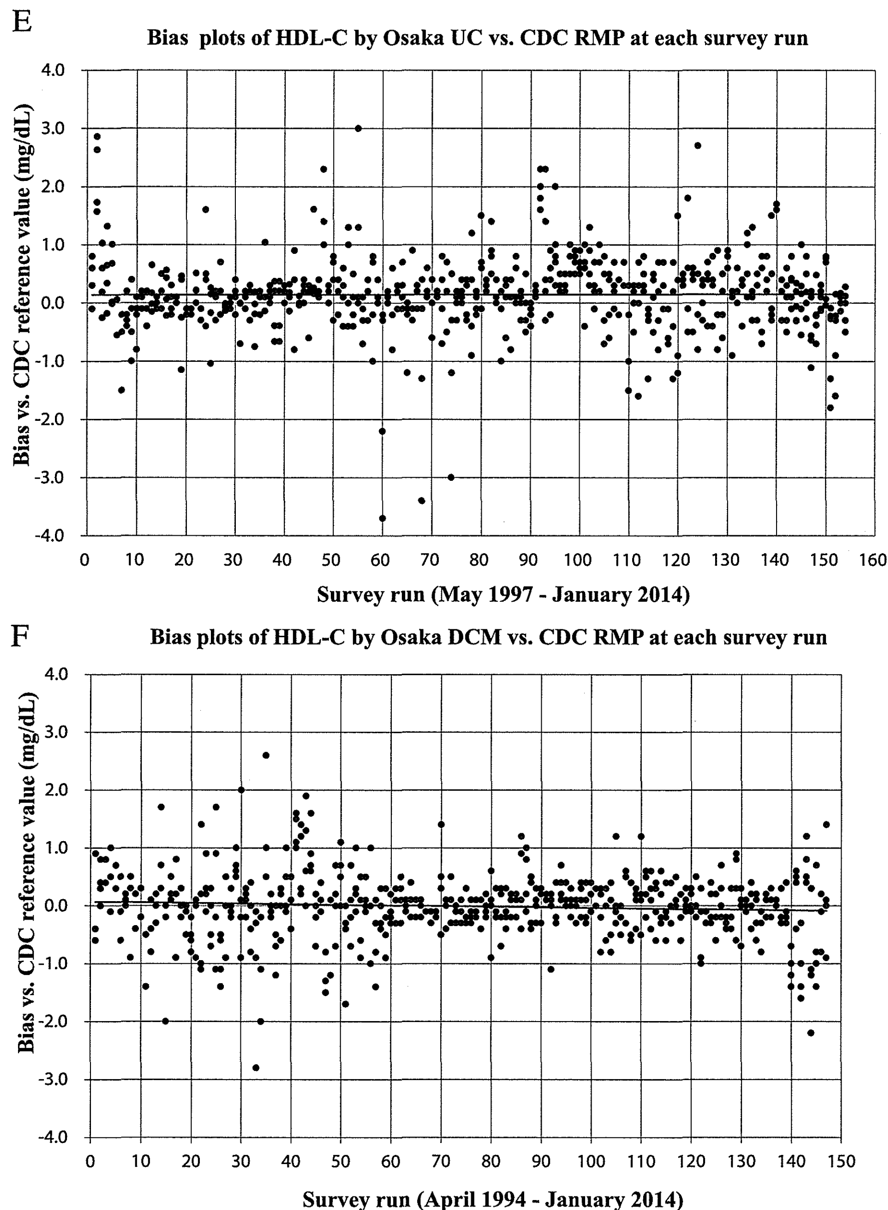

Fig. 3. E: Bias plots of HDL-C by Osaka UC vs. CDC RMP at each survey run. The y-axis indicates the bias (mg/dl) of Osaka UC compared to the CDC reference value and the x-axis indicates each survey run (May 1997–January 2014). CDC: The US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol. F: Bias plots of HDL-C by Osaka DCM vs. CDC RMP at each survey run. The y-axis indicates the bias (mg/dl) of Osaka DCM compared to the CDC reference value and the x-axis indicates each survey run (April 1994–January 2014). CDC: The US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol.

reached 55 and 65 mg/dl in 2012, which were markedly higher than those in Western countries [27]. We tentatively concluded that this could not be attributed to a drift in the standardization of HDL-C measurements in Japan because the increase was continuous over several time points when new assay reagents and systems were introduced. Furthermore, similar findings were reported for plasma apoA-I concentrations that were independently measured [26]. However, the underlying reasons for this phenomenon and its outcome on public health in Japan remain unknown. This is a unique and perhaps important finding for world public health; therefore, it should be extensively investigated in association with recent trends and changes in various aspects of Japanese lifestyles and medical/public health environments. It is also extremely important to monitor Japanese HDL-C levels carefully for years hereafter. Therefore, methods to measure HDL-C parameters must be established based on reliable standardization and stabilization for international consistency through CRMLN activities [16].

Since 1996, 7 Japanese reagent manufacturers have developed new homogeneous methods for HDL-C to replace the old precipitation-based methods [20]. These methods present new calibration challenges

Table 3
Performance criteria applied to clinical laboratory and manufacturer for HDL-C.

Parameter	Criterion
R^2	>0.975
Bias at 40 mg/dl	≤5%
Bias at 60 mg/dl	≤5%
Average % bias	≤5%
Average absolute % bias	≤5%
Among-run CV	≤4%
t-test of bias	Not significant at $\alpha = 5\%$
Within-method outliers	1 allowed
Between-method outliers	None allowed, but may eliminate one sample

because they use different principles that include detergents or surfactants to quantify HDL-C level. Homogeneous methods that do not require a sample pretreatment step are being introduced all over Japan and are used in many clinical laboratories. Based on the HDL Cholesterol Certification Protocol for Manufacturers (November 2002) by CRMLN, we conducted protocols 10 times every 2 years since 1996 using standardized DCM as a reference to Japanese manufacturers, and all manufacturers successfully met the performance criteria (Table 3). However, further accuracy improvements in homogeneous HDL-C methods will be required in several diseased samples derived from patients with dyslipidemia [28], which may require an increased use of the UC-RMP instead of the DCM. Unsolved issues associated with homogeneous methods remain and have yet to be examined in detail.

In conclusion, the UC method and the DCM for HDL-C at Osaka were found to be highly accurate, precise, and stable for many years, and assist reagent manufacturers in calibrating their products so that clinical and epidemiological laboratories can accurately measure HDL-C for patients and in research, calculate non-HDL-C, and estimate LDL-C with the Friedewald equation. DCM is a simpler equivalent reference method that has been consistent with CDC RMP for 20 years. However, the traceability of HDL-C should be accomplished by performing a method comparison with a fresh split sample because matrix interactions can severely affect HDL-C measurements.

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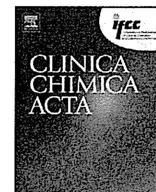

Total cholesterol performance of Abell–Levy–Brodie–Kendall reference measurement procedure: Certification of Japanese in-vitro diagnostic assay manufacturers through CDC's Cholesterol Reference Method Laboratory Network☆

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ABSTRACT

Background: Accurate measurement of total cholesterol (TC) is important for cardiovascular disease risk management. The US Centers for Disease Control and Prevention (CDC) and Cholesterol Reference Method Laboratory Network (CRMLN) perform Abell–Levy–Brodie–Kendall (AK) reference measurement procedure (RMP) for TC as a secondary reference method, and implement Certification Protocol for Manufacturers. Japanese CRMLN laboratory at Osaka performed the AK RMP for 22 years, and conducted TC certification for reagent/calibrator/instrument systems of six Japanese manufacturers every 2 years for 16 years. Osaka TC performance was examined and compared to CDC's reference values.
Methods: AK RMP involved sample hydrolysis, cholesterol extraction, and determination of cholesterol levels by spectrophotometry. The Certification Protocol for Manufacturers includes comparison with AK RMP using at least 40 fresh specimens. Demonstration of average bias ≤3% and total coefficient of variation ≤3% qualified an analytical system for certification.
Results: In the AK RMP used in the Osaka CRMLN laboratory, the regression equation for measuring TC was y (Osaka) = 1.000x (CDC) + 0.032 (n = 619, R^2 = 1.000). Six Japanese manufacturers had allowable performance for certification.
Conclusions: The AK RMP for TC measurement was accurate, precise, and stable for 22 years. Six Japanese manufacturers were certified for 16 years.

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1. Introduction

The association between elevated total cholesterol (TC), due to increased low-density lipoprotein cholesterol concentrations, and the risk of premature coronary heart disease (CHD) has been well documented [1–3]. CHD is the major cause of death in developed countries; accurate and reproducible TC measurements are of particular importance for correctly and consistently classifying individuals who are at increased risk for this disease, as is outlined in the clinical guidelines for the diagnosis, treatment, monitoring, and prevention of dyslipidema [4–7].

In 1988, the US Laboratory Standardization Panel [8,9] recommended that cholesterol measurements be standardized so that values are traceable to the US Centers for Disease Control and Prevention (CDC) reference measurement procedure (RMP) for cholesterol, which is a modification of Abell–Levy–Brodie–Kendall (AK) method [10,11]. As a result cholesterol tests performed in patient care as well as in clinical

☆ Disclaimer: The results and conclusions in this study are those of the authors and do not necessarily represent the views of the US Centers for Disease Control and Prevention.

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studies used to define clinical decision levels are standardized to the same AK RMP. This enabled the correct interpretation of cholesterol values and efficient implementation of clinical guidelines and public health efforts.

The AK RMP is linked to the National Institute of Standards and Technology (NIST) method for total serum cholesterol, which involves gas chromatography-isotope dilution mass spectrometry (GC-IDMS) [12–15]. The GC-IDMS RMP has higher specificity and selectivity than the AK RMP. Thus, results obtained with this method are not interchangeable with results from the AK RMP [10,14]. Because AK RMP-based clinical decision levels are currently being used in patient care, CDC continues to operate the AK RMP and standardizes clinical tests to this method. At the same time, it established a GC-IDMS RMP [14] to meet the increasing need for more specific and selective clinical measurements.

Epidemiological and large-scale clinical studies have been performed in Japan to investigate the risk of cardiovascular disease (CVD) using lipid measurements similar to studies conducted in Europe and the United States. The limitations of lipid measurement in Japan have historically been the comparability and accuracy of the assayed results. To overcome this limitation and to achieve traceable, accurate, and stable lipid measurements over time, an epidemiological study group at Osaka Medical Center has participated in the World Health Organization (WHO)-CDC Cooperative Cholesterol-Triglyceride Standardization Program since April 1975 [15–17]. The standardization of TC measurement at Osaka was achieved through the CDC-NHLBI Lipid Standardization Program in the 1970s and 1980s using Zak assays [18,19] and enzymatic methods [20,21], which are routinely used to analyze cholesterol levels in clinical laboratories in Japan. In 1991, the AK RMP for cholesterol was introduced to the epidemiological laboratory at Osaka, and it was standardized through the Cholesterol Reference Method Laboratory Network (CRMLN) from July 1992 to July 2014. TC certification has been performed by the CDC and CRMLN for reagent manufacturers using the Total Cholesterol Certification Protocol for Manufacturers [22]. For clinical laboratories, TC certification has been performed using the Certification Protocol for Clinical Laboratories [23]. As a result, most Japanese manufacturers and many clinical laboratories standardized TC measurements to provide traceability to the CDC's AK RMP. In 2002, the Osaka laboratory established a GC-IDMS method similar to CDC's RMP [24]. The AK RMP and GC-IDMS method have both been used continuously and simultaneously through regular CRMLN surveillance under the same measurement conditions since July 2012 to the present.

In this study, we present the accuracy and imprecision of the AK RMP obtained at Osaka during the course of 22 years. Moreover, we outline an evaluation of the accuracy, precision, and total error of reagent/calibrator/instrument systems of 6 Japanese reagent manufacturers that participated in the TC certification program for manufacturers [22] every 2 years for 16 years.

2. Materials and methods

2.1. Materials

In the CRMLN survey, all standardization pools for TC were created using sera that were prepared according to the Clinical and Laboratory Standards Institute Document C37-A [25], which defines blood collection, clotting and processing conditions. This suggests that no preservatives or additives were added nor was the material lyophilized. CRMLN survey pools include round robin samples that were provided from a participating CRMLN laboratory, and which included native specimens from patients and 12.1% (75 of 619 runs) of all samples. All survey pools were blinded to the CRMLN laboratories. Samples were shipped frozen from the CDC, and stored at −70 °C for subsequent analysis.

In the TC Certification Protocol for Manufacturers [22], participating manufacturers collected 40 or more fresh specimens from individual fasting donors. The cholesterol concentration of these specimens were distributed over a clinically meaningful range, as close as possible to the following target distribution: 20% of samples from 120 mg/dL to 180 mg/dL, 30% of samples from 181 mg/dL to 220 mg/dL, 30% of samples from 221 mg/dL to 260 mg/dL, and 20% of samples from 261 mg/dL to 400 mg/dL. The minimum amount of serum needed per sample for the AK RMP analysis is 1.5 mL. Manufacturers analyzed the specimens using their reagent/calibrator/instrument system over 20 runs, with 2 samples per run. To estimate imprecision, manufacturers should provide quality control (QC) single data obtained from 20 separate runs. The recommended concentration range for the QC material is 200 mg/dL to 240 mg/dL.

In the Certification Protocol for Clinical Laboratories [23], clinical laboratories analyzed two fresh samples in each of the three concentration regions; namely region 1: 100 mg/dL and 200 mg/dL, region 2: 200 mg/dL and 240 mg/dL and region 3: >240 mg/dL. The samples were assayed using the AK RMP at Osaka.

TC measurements were conducted at the Osaka Medical Center for Cancer and Cardiovascular Diseases between July 1997 and June 2001, at the Osaka Medical Center for Health Science and Promotion between July 2001 and March 2012, and at the National Cerebral and Cardiovascular Center at Osaka continuously since April 2012 (all laboratories are referred to as the Osaka laboratory).

2.2. Methods

2.2.1. AK RMP for TC at the Osaka CRMLN laboratory

The AK RMP for TC measurement is a modification of the extraction procedure by Abell et al. [10] and the original method was improved at the CDC laboratory [11]. We used a Digiflex (ICN, Biomedicals, Inc.) automatic pipettor for aspirating and dispensing standard solution, sample, and reagent. Current RMP consists of saponification of a 0.250 mL serum sample with alcoholic potassium hydroxide at 50 °C for 1 h, extraction for 20 min with hexane using a mechanical shaker in a horizontal position, evaporation of an aliquot of extract connected with a vacuum oven, and color development with Liebermann–Burchard reagent (mixed reagent of acetic anhydride, glacial acetic acid, and concentrated sulfuric acid) at 620 nm using a spectrophotometer (Beckman DU600 and DU800). The AK RMP is calibrated using the NIST Standard Reference Material (SRM) of pure unlabeled cholesterol (SRM 911c). The working standard solutions of cholesterol in alcohol consist of 25.0, 50.0, 100.0, 200.0, 300.0, and 400.0 mg/dL concentrations.

2.2.2. TC performance criteria applied to the CRMLN laboratory using AK RMP

TC performance criteria applied to the CRMLN lipid reference laboratory are summarized in Table 1. Precision was evaluated in terms of coefficient of variation (CV, %), and accuracy (%bias versus CDC reference value) was evaluated in terms of deviation (%) from the CDC reference value.

2.2.3. Statistical criteria of TC certification for manufacturers

Statistical criteria of TC certification for manufacturers are summarized in Table 2A. As a reference, statistical criteria of TC certification for clinical laboratories are summarized in Table 2B.

Table 1
TC performance criteria applied to CRMLN lipid reference laboratory using AK RMP.

Lipid	Precision criterion	Accuracy criterion
TC	Coefficient of variation ≤ 1%	Bias (deviation from CDC reference value) ± 1%

CRMLN: Cholesterol Reference Method Laboratory Network. AK RMP: Abell–Levy–Brodie–Kendall Reference Measurement Procedure. TC: total cholesterol.
CDC: Centers for Disease Control and Prevention.

Table 2A
Statistical criteria of TC certification for manufacturer.

Parameter	Criterion
r^2	>0.975
Bias at 200 mg/dl	≤3%
Bias at 240 mg/dl	≤3%
Average % bias	≤3%
Average absolute % bias	≤3%
Among-run CV	≤3%
Z-test of bias	Not significant at $\alpha = 5\%$
Within-method outliers	1 allowed
Between-method outliers	None allowed, but may eliminate one sample

Table 2B
Statistical criteria of TC certification for clinical laboratory.

Parameter	Criterion
r^2	>0.975
Bias at 200 mg/dl	≤3%
Bias at 240 mg/dl	≤3%
Average % bias	≤3%
Average absolute % bias	≤3%
Among-run CV	≤3%
t-test of bias	Not significant at $\alpha = 5\%$
Within-method outliers	1 allowed
Between-method outliers	None allowed, but may eliminate one sample

2.3. Statistical analysis

We used the protocol of NCCLS guideline EP9-A from the Clinical and Laboratory Standards Institute for bias estimation [26] and the STATA12 analysis program for all other calculations [27,28].

3. Results

3.1. Regression, accuracy and precision of TC by AK RMP at Osaka laboratory over time

In the AK RMP for TC measurement at Osaka, the CDC pooled sera with 219 different concentrations (lots) were analyzed among 619 survey samples with 165 survey runs, in which each survey run consisted of 3 to 4 different pools. There was one native sample from patients with low TC concentration ranged 27.7 to 83.4 mg/dL in the three to four provided pools: these samples were for the purpose of measuring the accuracy and precision of high-density lipoprotein cholesterol. The TC concentration ranged from 27.7 to 383.7 mg/dL: concentrations were analyzed during the course of 22 years between July 1992 and July 2014.

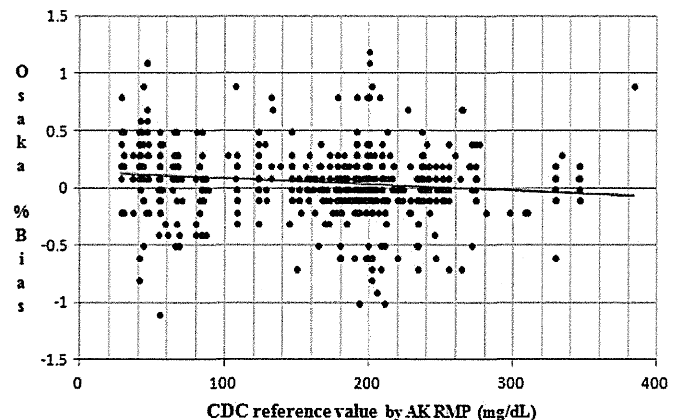

Fig. 1. Accuracy: %Bias plots of Osaka TC by AK RMP. Y-axis indicates the %bias vs. CDC reference value of Osaka TC by AK RMP and x-axis indicates CDC reference value by AK RMP (mg/dL). CDC: the US Centers for Disease Control and Prevention. AK RMP: Abell–Kendall reference measurement procedure. TC: total cholesterol.

In the scatter plots of %bias between Osaka (y) and CDC (x), $y = 1.000x + 0.032$ ($n = 619$, $R^2 = 1.000$). This means that 200 mg/dL at the CDC corresponds to 200.03 mg/dL at Osaka. The p-value and 95% confidence interval (CI) for the slope were $p < 0.001$ and (1.000, 1.001), respectively. The p-value and 95% CI for the intercept were $p = 0.502$ and (−0.061, 0.124), respectively (Table 3).

In the scatter plots of accuracy, %bias vs. CDC at Osaka, y (Osaka) $= -0.001 \times$ (CDC reference value) $+ 0.141$ (n: 619, $R^2 = 0.022$). The p-value and 95% CI for the slope were $p < 0.001$ and (−0.001, −0.000), respectively. The p-value and 95% CI for the intercept were $p < 0.001$ and (0.086, 0.195), respectively (Table 3). The Osaka laboratory met the acceptable accuracy goals within ± 1% compared to the CDC reference values for 99.0% of the samples (613 of 619) (Fig. 1., Table 3). The maximum %bias at Osaka AK RMP was +1.2% and the minimum was −1.1% among all 619 samples. The %bias between the reference values of the CDC and the measurements of the Osaka laboratory at a medical decision point of 200 mg/dL was only −0.06% at Osaka.

In the scatter plots of precision, CV(%) at Osaka, y (Osaka CV%) $= -0.001 \times$ (CDC reference value) $+ 0.321$ (n: 619, $R^2 = 0.093$). The p-value and 95% CI for the slope were $p < 0.001$ and (−0.001, −0.001), respectively. The p-value and 95% CI for the intercept were $p < 0.001$ and (0.286, 0.357), respectively (Table 3). The Osaka laboratory met acceptable precision goals < 1% at CV for 99.2% of the samples (614 of 619). The maximum CV at the Osaka AK RMP was 1.6% (Fig. 2.).

Table 3
Regression, accuracy and precision of TC by Osaka AK RMP over time.

Parameter	TC method	Number of samples	Pass rate	Slope (95%CI)	Intercept (95%CI)	R^2	Time period
Regression	AK RMP	619		1.000 (1.000, 1.001) p < 0.001	0.032 (-0.061, 0.124) p = NS	1.000	July 1992 to July 2014 (22 y)
Accuracy (as %Bias vs. CDC)	AK RMP	619	99.0%	-0.001 (-0.001, -0.000) p < 0.001	0.141 (0.086, 0.195) p < 0.001	0.022	July 1992 to July 2014 (22 y)
Precision (as CV)	AK RMP	619	99.2%	-0.001 (-0.001, -0.001) p < 0.001	0.321 (0.286, 0.357) p < 0.001	0.093	July 1992 to July 2014 (22 y)

AK RMP: Abell–Brodie–Levy–Kendall Reference Measurement Procedure. TC: total cholesterol.
For pass rate, TC accuracy criterion as %bias is ± 1% vs. CDC target value and TC precision criterion as CV is ≤1%.

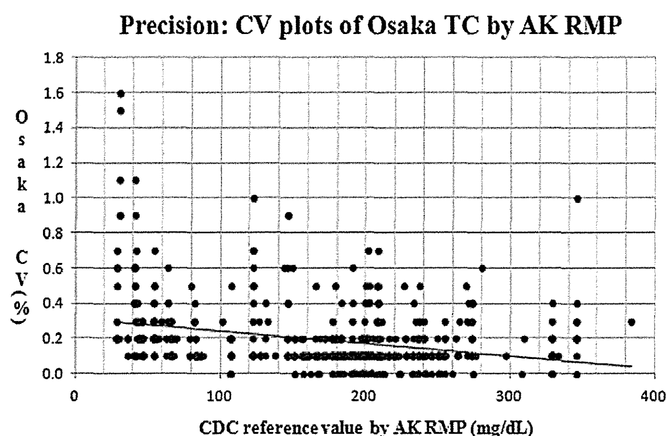

Fig. 2. Precision: coefficient of variation plots of Osaka TC by AK RMP. Y-axis indicates coefficient of variation (CV, %) of Osaka TC by AK RMP and x-axis indicates CDC reference value by AK RMP (mg/dL). CDC: the US Centers for Disease Control and Prevention. AK RMP: Abell–Kendall reference measurement procedure. TC: total cholesterol.

3.2. Accuracy, precision and total error of Japanese manufacturers conducted by the TC Certification Protocol for Manufacturers

Six reagent manufacturers in Japan were evaluated between 1996 and 2012 eight times according to the TC Certification Protocol for Manufacturers [22], with regard to their analytical systems consisting of a reagent/calibrator/instrument. Their accuracy (mean %bias versus reference value by AK RMP), precision (among-run CV), and total error (absolute mean %bias + 1.96 among-run CV) are presented in Table 4A, Table 4B, and Table 4C, respectively.

3.3. %Bias plots of TC by Osaka AK RMP at each run over time

Fig. 3 shows the %bias compared to the CDC reference value plots of the Osaka AK RMP at each run from a total of 165 runs for 22 years. The minimum value of the %bias was −1.1%, whereas the maximum value was 1.2%. The x-axis indicates the survey run number every 20 runs, and the y-axis indicates the %bias versus CDC reference value of the Osaka AK RMP. The acceptable criterion for the accuracy of TC was within ± 1.0% compared to the reference value of CDC. Each survey run consisted of three to four CDC pools, including round robin samples provided from the CRMLN laboratory for TC analysis.

4. Discussion

The CRMLN maintains robust reference measurement systems with high quality reference materials, measurement procedures, and continuous monitoring. Established protocols and guidelines within CDC's CRMLN certification program allows manufacturers to perform measurement comparisons between the test method and the reference method to assess performance accuracy and imprecision. As shown in earlier reports, CRMLN successfully applies this principle to complex analytes such as HDL- and LDL-cholesterol as well as to analytes such as total cholesterol. Reliable data allows for consistent patient monitoring, treatment management, and improved worldwide public health efforts for CVD.

The AK RMP as operated at the CDC Laboratory and the Osaka laboratory is highly accurate and reliable for over 20 years. Measurement results obtained by both laboratories show excellent agreement, precision and stability. This ensures assay manufacturers calibrated by the Osaka CRMLN laboratory can produce measurement results that are highly accurate and comparable over time.

The main purpose of the CRMLN laboratories is to work with manufacturers to certify the accuracy and precision of cholesterol measurements of reagent/calibrator/instrument systems used in clinical laboratories [29]. This is in agreement with the US Laboratory Standardization Panel, which suggests that standardization is most effectively achieved through the manufacturers of analytical instruments and reagents [8,9]. Between 1996 and 2012, six reagent manufacturers in Japan were certified eight times with regard to their analytical systems consisting reagent/calibrator/instrument [22]. Standardization of 2,122 Japanese clinical laboratories has been performed by the Certification Protocol for Clinical Laboratories [23] between 1993 and 2014, and 98.2% of these participants met the certification criteria, which were derived from clinical needs. This high pass rate with clinical laboratories suggests that calibration of assay manufacturers by CRMLN laboratories is highly successful and effective. Lipid standardization activities have improved the accuracy of TC measurements [30]. All manufacturers and clinical laboratories with current certification are listed on CDC's-CRMLN web site (http://www.cdc.gov/labstandards/crmln.html).

Table 4A
Accuracy (mean %bias) of 6 Japanese manufacturers conducted by TC Certification Protocol for Manufacturers.

	Certification year for TC								
Manufacturer	1996	1997	1998	2002	2004	2006	2008	2010	2012
A	0.6		-0.8	-0.4	-0.1	1.0	-0.3	-0.3	1.3
B	-1.2		-0.6	1.6	-0.4	1.4	1.0	-0.3	-0.1
C	1.0		-1.3	-1.0	0.6	-0.4	1.1	-0.1	-0.9
D	2.1	1.2		-1.1	-0.1	0.7	0.1	-0.5	0.5
E		−2.2		0.2	0.2	−0.2	1.8	0.5	−0.1
F		1.2		0.4	−0.4	0.0	0.5	0.3	0.0

Accuracy criterion: mean % bias ≦3% unit: %.

Table 4B
Precision (among-run CV) of 6 Japanese manufacturers conducted by TC Certification Protocol for Manufacturers.

	Certification year for TC								
Manufacturer	1996	1997	1998	2002	2004	2006	2008	2010	2012
A	0.5		0.6	0.6	0.6	0.8	0.4	0.5	0.5
B	0.7		0.7	0.7	0.7	1.2	0.6	0.6	0.7
C	0.6		0.7	0.5	0.6	0.5	0.6	0.5	0.5
D	0.6	0.7		0.5	0.4	0.3	0.4	0.4	0.4
E		0.4		0.5	0.7	0.5	0.4	0.8	1.0
F		1.5		0.8	0.7	0.5	0.5	0.4	1.0

Precision criterion: among-run CV ≦ 3% unit: %.

Table 4C
Total error of 6 Japanese manufacturers conducted by TC Certification Protocol for Manufacturers.

	Certification year for TC								
Manufacturer	1996	1997	1998	2002	2004	2006	2008	2010	2012
A	1.5		2.0	1.5	1.3	2.6	1.1	1.2	2.3
B	2.6		2.1	3.0	1.7	3.9	2.3	1.5	1.4
C	2.2		2.7	2.0	1.8	1.4	2.3	1.0	1.8
D	3.3	2.7		2.2	0.8	1.4	0.9	1.3	1.3
E		3.0		1.3	1.6	1.3	2.6	2.1	2.1
F		4.2		1.9	1.8	1.0	1.5	1.1	2.0

Total error (absolute mean %bias + 1.96 among-run CV) criterion: ≦8.9% unit: %.

The AK RMP was proposed more than 60 years ago and is considered the secondary reference method for analyzing cholesterol. It has been used internationally as a reference procedure for measuring cholesterol at CDC, CRMLN laboratories, and in many epidemiological research institutes worldwide. Despite its widespread use, the AK method has limitations such as complex operations, the requirement of skilled technicians, use of hazardous reagents, and interferences related to the measurement of reactive substances other than cholesterol. These limitations can be minimized with current GC-IDMS RMPs.

Current clinical and public health decision points are based on measurements standardized to the AK RMP. Because results obtained with this RMP are not interchangeable with results obtained with GC-IDMS RMPs, and a reliable relationship between both types of RMPs has not been established yet. The AK RMP continues to be used to standardize tests in patient care and public health. At the same time, CDC together with CRMLN laboratories including Osaka laboratory is working to further establish mass spectrometry-based RMPs for TC and other lipid and lipoproteins [14,31–33]. Mass spectrometry-based RMPs for TC and triglycerides are already part of the CRMLN. This will help the clinical and public health communities to generate new data and clinical decision points that are linked to new more specific mass spectrometry-based RMPs.

5. Conclusions

We presented the performance of TC measurements using the AK RMP over the past 22 years and demonstrated the accuracy, precision, and long-term stability of this method. As examples of AK RMP application, we presented six reagent manufacturers in Japan that successfully certified their reagent/calibrator/instrument systems and participated in the TC Certification Protocol for Manufacturers for 16 years. The clinical laboratories in Japan demonstrated high achievement rates for TC certification. This accomplishment is one example on how CRMLN is improving clinical testing of cholesterol and other blood lipids not only in Japan but worldwide. These data as well as data shown in previous reports demonstrate that the same standardization principles can be applied to analytes such as TC as well as highly complex analytes such as HDL- and LDL-cholesterol.

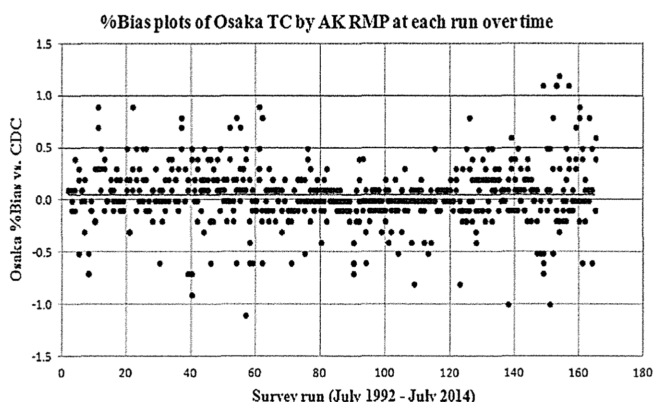

Fig. 3. %Bias plots of Osaka TC by AK RMP at each run over time. Y-axis indicates Osaka %bias vs. CDC reference value and x-axis indicates each survey run from total 165 runs (July 1992 - July 2014). CDC: The US Centers for Disease Control and Prevention. AK RMP: Abell–Kendall reference measurement procedure. TC: total cholesterol.

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Conflict of interest

No authors have any financial, personal or professional relationships associated with other people or organizations.

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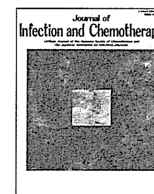

Original article

Genotyping of *Acinetobacter baumannii* strains isolated at a Japanese hospital over five years using targeted next-generation sequencing

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ABSTRACT

Acinetobacter baumannii is a Gram-negative bacterial agent involved in nosocomial infections. In this five-year retrospective study, phylogenetic relationships among carbapenem-resistant *A. baumannii* strains that were isolated at Teikyo University Hospital in Tokyo metropolis, Japan, were explored. A panel of 72 carbapenem-resistant *A. baumannii* strains that isolated from January 2006 until August 2010 was studied. Next-Generation sequencing (NGS) was employed to perform large-scale genotyping of these isolates. They were separated, according to the time of isolation, into two genetically distinct groups, one correspondent to strains of the outbreak reported to local public health department in 2010 and the other contained strains from earlier isolations, suggesting different origins of the isolates. Moreover, taxa in each group showed two main clustering patterns. Multilocus sequence typing (MLST) study on 8 isolates from the last outbreak showed that they were from one sequence type, 92, displaying less discriminatory power comparing to large-sequence typing. The clonal lineage profiles produced in this retrospective study will be used as a reference database to compare future isolations of *A. baumannii*. This study demonstrates the power of NGS in conducting epidemiological researches, allowing a high resolution genotyping.

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1. Introduction

Acinetobacter baumannii is a Gram-negative bacterial agent that has emerged internationally as an increasingly important nosocomial pathogen [1]. It colonizes on skin, respiratory tracts of patients, and medical instruments [2]. Antimicrobial resistance among *Acinetobacter* isolates has been increasing, limiting therapeutic options for patients, especially with resistance against carbapenem class drugs. *A. baumannii* causes outbreaks and health care-associated infections including bacteremia, pneumonia, meningistis, and wound infection.

Good managements of health care facilities on drug-resistant organisms should include rapid identification, screening for drug resistances and typing approaches. Genotypes of this organism has commonly been identified based on multilocus sequence typing (MLST) and pulse-field gel electrophoresis (PFGE) [3]. Discriminability of PFGE is better compared to MLST which is suitable for separating epidemiologically unrelated isolates, however, PFGE is technically demanding and labor-intensive approach [4]. On the

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contrast, targeted next-generation sequencing technologies increasingly are applied to cover such demands, which proved to show a more resolution in detecting clonal lineage of isolates collected during outbreaks [5].

In order to study clonal lineage of carbapenem-resistant *A. baumannii* isolates, we used the Pathogenica HAI BioDetection system (Pathogenica Inc., Boston, MA, USA), a Next-Generation sequencing (NGS) based technology. Adopted to the Ion Torrent PGM (life technologies), every reaction contains thousands of molecular inversion probes that capture short DNA sequences from loci at genomes and plasmids of a set bacterial panel of 12 nosocomial infectious pathogens including *A. baumannii* (for more details refer to Ref. [6]). In addition, it screens for the presence of 18 drug-resistance genes including OXA, CARB, SHV, and AmpC β-lactamases, however, correlation between susceptibilities and presence of drug-resistant genes has not been explored yet in the current version of the kit. These isolates are correspondent to 72 different clinical cases, one strain from one patient, and were collected at Teikyo University Hospital in Tokyo metropolis over a course of 5 years from 2006 onward, which marked the occurrence of an outbreak by *A. baumannii* reported to local public health department in 2010 [7].

2. Material and methods

2.1. Strains and media

The bacterial panel explored in this phylogenetic study included non-duplicated clinical strains of carbapenem-resistant *A. baumannii* that were isolated at Teikyo University Hospital in different periods. Twenty-three strains were isolated from 2006 through 2009, while the remaining 49 strains were isolated during the outbreak occurred around 2010. Isolations were carried out on BTB agar blue supplemented with 2 μg/ml of cefotaxime or on CHROMagar™ *Acinetobacter* at 35 °C for 48 h or 24 h, respectively. These isolates were recovered from blood, sputum, stools, urine, bronchoalveolar lavage, pus or pharyngeal mucus specimens. Primary identification and susceptibilities to antibiotics were examined using MicroScan kit version 3.5 (Siemens). The study design was approved by an ethics review board of Teikyo University (TEI–I–RIN–11–122).

2.2. Multilocus sequence typing

Relatedness among randomly chosen 8 strains isolated during the last outbreak was explored by MLST scheme depending of seven housekeeping genes, i.e., *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD*. These strains are highlighted in the Fig. 1. PCR primers, conditions and analyses were as described previously [8]. Amplicons were sequenced using an ABI Prism 3130xl sequence analyzer (Applied Biosystem), and sequences were compared with the *A. baumannii* database at the MLST website [9].

2.3. Large-scale DNA sequencing

Total DNA was extracted from isolates using InstaGene Matrix Kit (BIO-RAD) in accordance with manufacturer's instructions. DNA collections were stored at −80 °C until they were used. For more precise identification and molecular typing, DNA libraries correspondent to the above isolates were generated by HAI BioDetection Kit according to manufacturer's instructions, followed by cleaning up barcoding reactions using AMPure XP Purification Kit (Beckman Coulter). Libraries were processed through Ion PGM template preparation and sequencing 200 kit v2 (Life Technologies) before to be loaded into Ion 316™ Chip v2.

2.4. Bioinformatic analyses

Reads resulted from each Ion PGM run were automatically analyzed with Pathogenica Software version 1.2.0 provided by Pathogenica. It is an automated process where barcoded sequences are assorted and consensus sequences that showed minimal level of length and quality are reported. These sequences were combined and used to produce a phylogenetic tree according to the neighbor-joining method using the software MEGA (version 6.0) [10]. Length of sequences for the isolates ranged between 4 kb and 6 kb, however, when drawing the phylogeny, all positions with less than 5% site coverage were eliminated.

3. Results and discussion

Detection of relatedness among isolates during nosocomial outbreaks has great epidemiological significance. However, typing methods are restricted, mostly time-consuming and labor-intensive [4]. In this retrospective study, carbapenem-resistant *A. baumannii* strains isolated during the outbreak occurred around 2010 and during earlier isolations were classified using large-scale DNA typing based on HAI BioDetection kit.

Pilot study included 8 strains isolated during the last outbreak of *A. baumannii* at Teikyo University Hospital, revealed that they were all of sequence type 92 according to the MLST database website [9]. However, these results do not comprise too much information of epidemiological importance. MLST approach is known for its discriminatory power with epidemiologically unrelated strains only, which is not usually the case in nosocomial outbreaks.

In order to implement more useful approach for outbreak investigation and control, we applied large-scale genotyping based on HAI BioDetection kit. As shown in Fig. 1, taxa dispersed onto two main groups, designated as Group "A" and Group "B". Group "A" included taxa that are correspondent to isolates from the outbreak occurred around 2010. It also subdivided into two main clustering patterns, A1and A2, that contained the clinical strains isolated from August 2009 until August 2010. On the other hand, Group "B" contained taxa correspondent to strains of early isolations, except for the clinical strain TURA070 (August 2010). It also subdivided into two clusters, B1 and B2. The cluster B1 contained the isolates of 2009 except the taxa TURA014 and TURA016, which were isolated in August 2008 and November 2008, respectively. However, the cluster B2 included the isolates of 2006 but the taxa TURA013 (April 2008), TURA015 (September 2008), TURA017 (December 2008) and TURA070.

While MLST did not discriminate among 8 isolates of the same outbreak, HAI BioDetection Kit provided data of large-scale sequencing, allowing production of phylogenetic tree with greater resolutions (Fig. 1). Moreover, the current study shows that carbapenem-resistant *A. baumannii* strains isolated at Teikyo University Hospital over a course of five years are from two genetically distinct groups (Fig. 1). The Group "B" showed a clustering pattern mainly reflects the year of isolation, i.e., B1 contained isolates of 2009 and B2 included isolates of 2006, suggesting two different origins of the pathogen. Moreover, the Group "A" contained the clinical strains isolated from August 2009 until August 2010 that dispersed into two main clusters, A1 and A2. Although the 8 isolates that were typed with MLST scheme accommodated in the genetic group A, they were dispersed onto its two main clusters A1 and A2 produced by HAI BioDetection Kit, implying a more powerful discriminability.

The HAI BioDetection Kit can capture plasmids and genomic sequences, providing a discriminatory power to separate even closely related strains. However, when drawing the phylogenetic tree, all positions with less than 5% site coverage were eliminated.