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Implementation of Cellulomonas Cholesterol Oxidase for Total Serum Cholesterol Determination by the Endpoint Method

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Cellulomonas has been shown to be a good source of cholesterol oxidase in addition to *Streptomyces* for serum cholesterol determination by the endpoint method, inexpensive in cost, and showing excellent performance. For clinical use, we have assessed the reliability of *Cellulomonas* reagent for cholesterol determination. We constructed the user-defined endpoint methods on three automated analyzers. The analytical performances (linearity, precision, recovery, interference, stability, and comparison with the standardized method) of *Cellulomonas* cholesterol reagents were evaluated and compared to those of *Streptomyces* reagents. Linearity (18.1–23.3 mmol/L) and stability of reagents (6–11 weeks) depended on the analyzers being used. The average within-run and between-day % coefficients of variation (CVs) ranged from 1.44 to 2.45 and 1.98 to 2.99, respectively, and were within National Cholesterol Education Program analytical criteria ($\leq$3%). All assays using both reagents compared favorably with the commercial method and appeared accurate near the clinical decision cut-points. Hemoglobin concentration at 7.5 g/L only affected the assay using single wavelength measurement. Bilirubin decreased in serum cholesterol recovery while lipemia generated a positive interference with all methods. *Cellulomonas* enzyme is analytically reliable when used for serum cholesterol determination by the endpoint method. Its analytical performance is equivalent to *Streptomyces* enzymes and meets the analytical goals. It has an advantage over the other enzymes in that it does not ship in the frozen state. J. Clin. Lab. Anal. 22:50–58, 2008.

Key words: serum cholesterol; *Cellulomonas*; *Streptomyces*; cholesterol oxidase; enzymatic endpoint method; implementation; coronary heart disease

INTRODUCTION

Measurement of cholesterol is widely used in clinical laboratory for assessment the risk of coronary heart disease (1). Furthermore, all methods for low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) determination included a separation of specific lipoprotein particles and followed by direct measurement of the total amount of cholesterol they carry (2,3). All methods used to measure cholesterol for lipid testing need to be accurate and precise (4). With the increasing interest in measuring lipids of the peoples of tropical and subtropical countries, factors relating to cost and ease of shipping need to be considered. In many tropical countries, it is difficult or very expensive to ensure shipment of reagents in the frozen condition. *Cellulomonas*-derived cholesterol oxidase has an advantage over other sources in that it does not require shipment in the frozen state. It has yet to be determined whether cholesterol oxidase derived from this source would suffice as an acceptable reagent by conventional cholesterol methods.

Most popular determination of serum cholesterol is the enzymatic method, which involves the use of three

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enzymes, cholesterol esterase (EC 3.1.1.13), cholesterol oxidase (EC 1.1.3.6), and peroxidase (EC 1.11.1.7) (5). The assay is generally performed as an endpoint or a kinetic method (6). Advantages of the kinetic method over the endpoint method are shorter analysis time, reduced effects of interfering substances, and elimination of sample blank measurement. The endpoint method's main advantage over the kinetic method is its insensitivity to minor changes in reaction conditions.

According to previous studies (7), we introduced *Cellulomonas* as a new source of cholesterol oxidase besides *Streptomyces*, for determining serum cholesterol by the enzymatic endpoint method. It is superior to enzymes from *Brevibacterium* and *Pseudomonas fluorescens* sources, because of its lower cost and less hemoglobin interference. To facilitate the reliability of this enzyme, one must construct user-defined methods on the different automated analyzers that are widely used in routine clinical laboratories. The analytical performances of the reagent containing *Cellulomonas* enzyme was compared to *Streptomyces* for serum cholesterol determination by the endpoint method.

MATERIALS AND METHODS

Equipment

We used the Vitalab Selectra Analyzer (E. Merck, Dramstadt, Germany), the Mega Analyzer (E. Merck) and the Dimension RxL (Dade Behring, Newark, DE) for the implementation of endpoint cholesterol assay. The Vitalab Selectra is an analyzer with small and medium throughput (up to 180 tests per hr) while the Dimension RxL and Mega are large, high-sample volume automated analyzers (throughput 500–800 tests per hr). The analyzers differ in the principle of photometric measurement as shown in Table 1.

Chemical

All enzymes and chemicals were obtained from the Sigma Chemical (St. Louis, MO), except the 4-aminophenazone was obtained from BDH (Dorset, England).

Reagents

We prepared the cholesterol reagents by dissolving *Cellulomonas* cholesterol oxidase (300 U/L) or *Streptomyces* cholesterol oxidase (200 U/L), cholesterol esterase from bovine pancreas (200 U/L), peroxidase (10,000 U/L), sodium cholate (3 mmol/L), 4-aminophenazone (0.5 mmol/L), phenol (20 mmol/L), and Triton X-100 (2 mL/L), in phosphate buffer (0.1 mol/L, pH 7.0).

TABLE 1. Description of the user-defined cholesterol methods on various analyzers

	User-defined parameters		
	Vitalab Selectra	Mega	Dimension
Reaction mode	Endpoint	Endpoint	Endpoint
Incubate reagent time (min)	1.3	0.1	1.0
Incubation times (min)	11.5	9.0	10.0
Total analysis time (min)	12.8	9.1	11.0
1° wavelength (nm)	505	500	510
2° wavelength (nm)	–	604	600
Sample volume (μL)	3	3	3
Chase volume (H_2O; μL)	–	–	20
Reagent volume			
R 1 (μL)	300	300	300
R 2 (μL)	–	–	–
Chase (H_2O; μL)	–	–	40
Principle of measurement	One point	One point	Two points P1: −10 sec P2: 600 sec
Calibration type	–	Linear	Linear
Calibrator(s)	1 level	1 level	3 levels

We used the commercial Dimension RxL's endpoint cholesterol method (8) as the reference comparison method. Our laboratory was standardized through the Center for Disease Control and Prevention (CDC) Lipid Standardization Program. The bias and precision for cholesterol measurement were within the acceptable criteria (<3%).

Procedures

The clinical performance of the user-defined methods using two reagents was evaluated.

Linearity and reportable range

To assess the linearity of each method, we used a set of sera with cholesterol ranging from 0.5 to 23.3 mmol/L, which was prepared accurate in equally spaced concentrations from zero to 2.6 mmol/L (0.52 mmol/L spaced intervals) and 2.6 to 23.3 mmol/L (2.6 mmol/L spaced intervals). The absorbances were plotted vs. cholesterol concentrations. Linearity was evaluated using the NCCLS EP6-A guideline and the linear region reported as the reportable range (9).

Imprecision

We selected sera with low (~2.8 mmol/L), middle (~5.0 mmol/L), and high (~10.0 mmol/L) cholesterol concentration. The within-run (20 replicates in the same run) and between-run (20 consecutive days) imprecisions were determined in each serum sample. The means,

standard deviations (SD), and coefficients of variation (CV = SD/mean × 100%) were calculated.

Recovery

We performed the recovery study by mixing sera containing low (2.67 mmol/L), middle (4.93 mmol/L), and high (9.20 mmo/L) cholesterol concentration in the ratio of 1:1 (low + middle, low + high, and middle + high). We calculated the recovery of cholesterol from the test reagents compared with the Dimension RxL's method in percentage.

Comparison

The fresh sera, normal in appearance (n = 100) were analyzed in parallel with the test methods and the commercial Dimension RxL cholesterol assay (8). The regression equations, correlation coefficients, bias, and standard error of the estimate ($S_{y/x}$) values obtained between the methods were calculated. We also performed the relative difference plot to judge the clinical acceptability of the implemented method by plotting the % relative difference (test method minus Dimension RxL's method, divided by Dimension RxL method and multiplied by 100) vs. the average value of two methods.

Interference study

We prepared the sets of hemolyzed (hemoglobin ranging from 0 to 15.0 g/L), icteric (bilirubin ranging from 0 to 1368 μmol/L), and turbid samples (absorbance at 670 nm of 0–2.334). A 1:2 dilution of each interference sample with pooled serum of low, middle, and high cholesterol levels was made to obtain three sets of various degrees of interfering substances. Cholesterol in each sample's set was determined by using the experimental cholesterol reagents. Interfering substances are considered significant when their effects disturb the results by ±9% or more according to NCEP total analytical goal for cholesterol determination (4).

Stability

Stability of each cholesterol reagent was investigated by analyzing the middle (4.7 mmol/L) and high (8.8 mmol/L) cholesterol samples for 3 months. The reagents were storage in a refrigerator (2–8°C).

RESULTS

The linearity of the implemented cholesterol assays using *Streptomyces* and *Cellulomonas* enzyme is illustrated in Fig. 1. We observed the sensitivity and the linearity patterns obtained from *Streptomyces* reagent (Fig. 1a, c, e) quite similar to those from *Cellulomonas* enzyme (Fig. 1b, d, f). The reportable range for the assay on Vitalab Selectra (1.04–20.7 mmol/L), Mega (1.04–18.1 mmol/L), and Dimension RxL (1.29–23.3 mmol/L) was proven by the polynomial method (NCCLS EP6-A) (9).

The range of the average within run imprecision was 1.44–2.45% (mean, 1.84%) and 1.44–2.19% (mean, 1.74%) for the *Streptomyces* and *Cellulomonas* cholesterol reagent, respectively. For the day-to-day run imprecision, the average range was 2.41–2.99% (mean, 2.66%) and 1.98–2.82% (mean, 2.35%), respectively. All precision values were acceptable and within analytical goal (%CV < 3.0) recommended by the National Cholesterol Education Program (NCEP) (4).

The average percent of the analytical recovery of implemented methods was ranging from 95.6% to 103.4% and 96.3% to 102.6% by using *Streptomyces* and *Cellulomonas* cholesterol reagent, respectively.

Results of correlation studies of all implemented methods vs. the CDC-standardized cholesterol method (commercial Dimension RxL cholesterol assay) (8) by least-square linear regression analysis are shown in Table 2. All methods show excellent correlation (r > 0.995) and the range in values for the 95% confidence interval of slope and y-intercept were very tight.

Fig. 2 shows the relative different plots between the implement assays and the reference comparison method. We used total analytical error equal to 9% recommended by NCEP (4) for assessment of the agreement between the two methods. We found that all results of test methods were within the total error criteria for cholesterol determination.

To evaluate the bias error of the methods using the experiment cholesterol reagents, we also analyzed the reference samples from CDC Lipid Standardization Program Part III on the Dimension RxL analyzer. In each method, four different samples (cholesterol concentration ranged from 3.92 to 5.10 mmol/L) were analyzed six times on three runs. The % biases (the differences of the results by the CDC and each reagent) were calculated and are tabulated in Table 3. The mean bias was ranged from −0.57 to 0.15% and −0.31 to 0.68%, for the methods using *Streptomyces* and *Cellulomonas* enzymes, respectively, which fell within NCEP guidelines (≤3%).

Table 4 tabulates the analytical error of the implemented methods. We estimated the systematic error as a percentage of each cholesterol assays at the clinical decision cut-point of serum cholesterol (5.2 and 6.2 mmol/L) from the regression analysis. The systematic error ranged from −0.8 to 1.3% and −0.7 to 3.0%

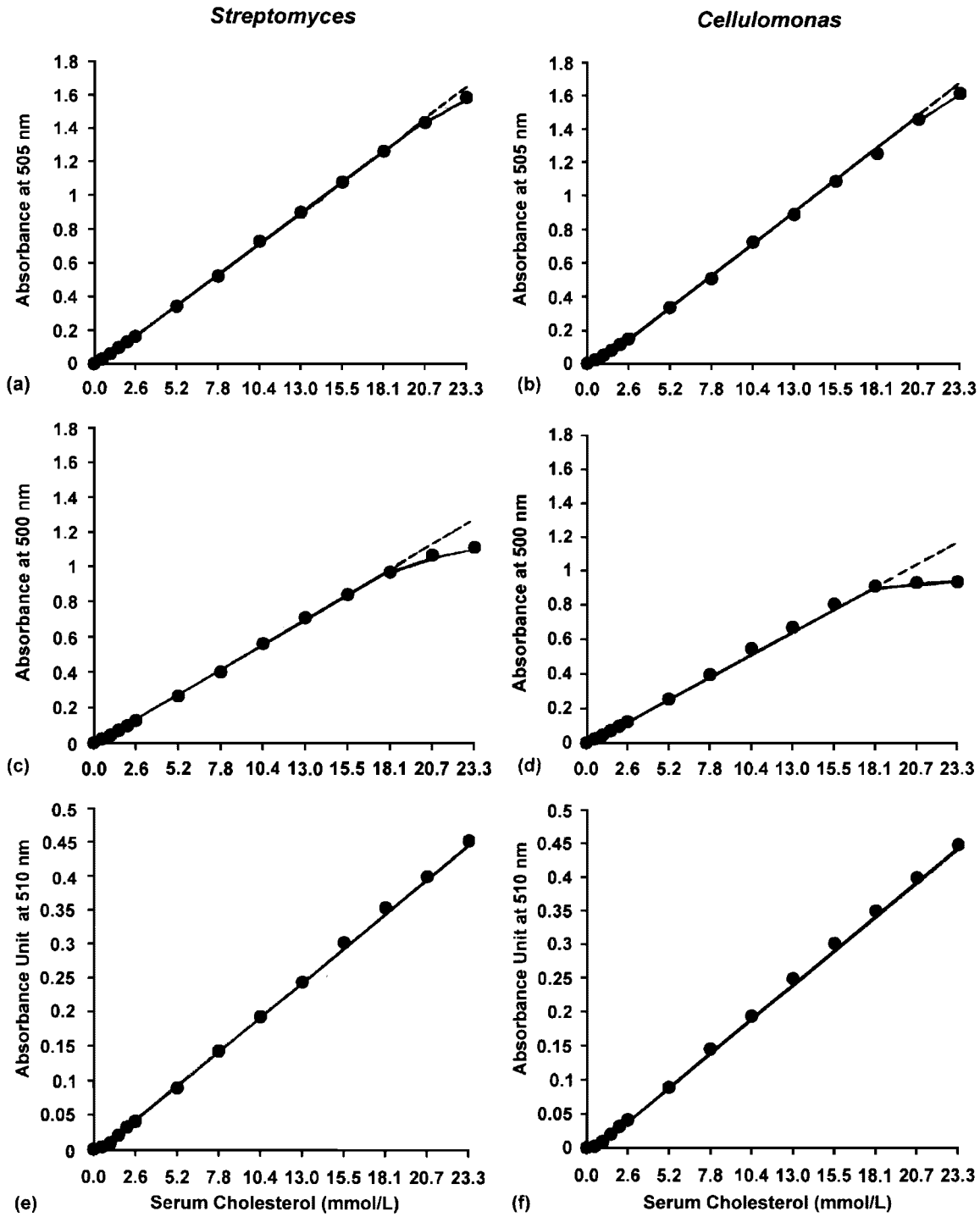

Fig. 1. Linearity of serum cholesterol obtained from *Streptomyces* (**a**, **c**, **e**) and *Cellulomonas* (**b**, **d**, **f**) cholesterol reagents by using Vitalab Selectra (a and b), Mega (c and d), and Dimension RxL (e and f) analyzers.

for the methods using *Streptomyces* and *Cellulomonas* enzymes, respectively.

The assays showed no interference from hemoglobin except at a concentration of 7.5 g/L that gave a positive bias on serum cholesterol level by the methods on Vitalab Selectra. Turbidity of degree one plus (absorbance >0.389 at 670 nm) gave positive bias on cholesterol for all experimental methods. On the other

TABLE 2. Correlation of implemented methods on various instruments and reference comparison assay (Dimension RxL method)

Method	Mean (mmol/L)	r	$S_{y/x}$	Bias	Coefficients	
					Slope (95% CI)	Intercept (95% CI)
Vitalab Selectra						
Streptomyces	5.663	0.998	0.137	0.023	1.058 (1.044–1.071)	−0.301 (−0.381–−0.222)
Cellulomonas	5.787	0.995	0.218	0.147	1.064 (1.043–1.085)	−0.214 (−0.341–−0.087)
Mega						
Streptomyces	5.608	0.997	0.173	−0.032	1.039 (1.022–1.056)	−0.252 (−0.353–−0.151)
Cellulomonas	5.584	0.996	0.189	−0.056	1.020 (1.002–1.038)	−0.169 (−0.279–−0.059)
Dimension RxL						
Streptomyces	5.733	0.998	0.142	0.093	1.004 (0.990–1.018)	0.070 (−0.014–0.152)
Cellulomonas	5.772	0.995	0.206	0.132	0.995 (0.975–1.015)	0.158 (0.038–0.278)
Commercial method	5.640					

TABLE 3. The analytical bias of implemented Dimension RxL cholesterol assays using *Streptomyces* and *Cellulomonas* cholesterol reagent

CDC sample	Cholesterol (mmol/L)[a]	Bias (%)	
		Streptomyces	*Cellulomonas*
1	3.92	0.15	0.68
2	4.09	0.14	0.37
3	4.43	−0.13	0.61
4	5.10	−0.57	−0.31
Average		−0.10	0.34

[a]The reference value was assigned from the Abell-Kendall reference method (10).

TABLE 4. The analytical error of the implemented cholesterol assays using *Streptomyces* and *Cellulomonas* cholesterol reagent

Cholesterol assays	Systematic error (%)[a]		Imprecision (%)[b]
	5.2 mmol/L of cholesterol	6.2 mmol/L of cholesterol	
Vitalab Selectra			
Streptomyces	0.2	1.2	2.6
Cellulomonas	2.0	2.6	2.0
Mega			
Streptomyces	−0.8	0.0	3.0
Cellulomonas	−1.3	−0.7	2.8
Dimension RxL			
Streptomyces	1.3	1.1	2.4
Cellulomonas	3.0	2.6	2.3

[a]Systematic error was calculated at the clinical decision cut-point of serum cholesterol (5.2 and 6.2 mmol/L) from the regression equations.
[b]Imprecision error was calculated from 20 consecutive day measurements of the implemented methods.

hand, bilirubin gave a negative bias; most methods experienced interference when the bilirubin concentration exceeded 171.0 μmol/L.

Fig. 3 shows the stability pattern of each reagent obtained form the normal and high cholesterol samples on various analyzers. The stability of reagents on Mega (6 weeks) was less than Vitalab Selectra (10–11 weeks) and Dimension RxL (11 weeks). This may be due to the shorter analysis time using in the Mega (around 9 min) than the Vitalab Selectra (12.8 min) and the Dimension RxL (11 min) analyzers (Table 1).

DISCUSSION

This study provides evidence that *Cellulomonas*-derived cholesterol oxidase meets acceptable criteria for the determination of cholesterol by an endpoint method. This enzyme reagent differs from others in that it does not require frozen shipment and is still inexpensive, important factors for laboratories in tropical and subtropical countries.

Cholesterol may be quantitatively determined either by an endpoint (5–7) or a kinetic method (11,12). Advantages of the endpoint over the kinetic method are better precision and lower reagent cost (6). The imprecision of the endpoint method was within NCEP guidelines, whereas imprecision of the kinetic method was not. The cost of enzyme used in the reagent for the endpoint method is one-fifth that of the kinetic method. However, the kinetic method shows less interference and shorter analysis time. We have previously demonstrated that the endpoint method proved the best assay for central laboratory determination because its accuracy and precision meet the currently established analytical performance goal (6).

Streptomyces, *Pseudomonas fluorescens*, *Cellulomonas*, and *Brevibacterium* cholesterol oxidase could be used for serum cholesterol determination by the endpoint method (7). The differences in the properties of enzyme make *Pseudomonas fluorescens* and *Brevibacterium* cholesterol oxidase inferior to *Streptomyces* and *Cellulomonas*. The *Pseudomonas fluorescens* source is susceptible to hemoglobin interference while the *Brevibacterium* enzyme activity requirement is at least five-fold higher than

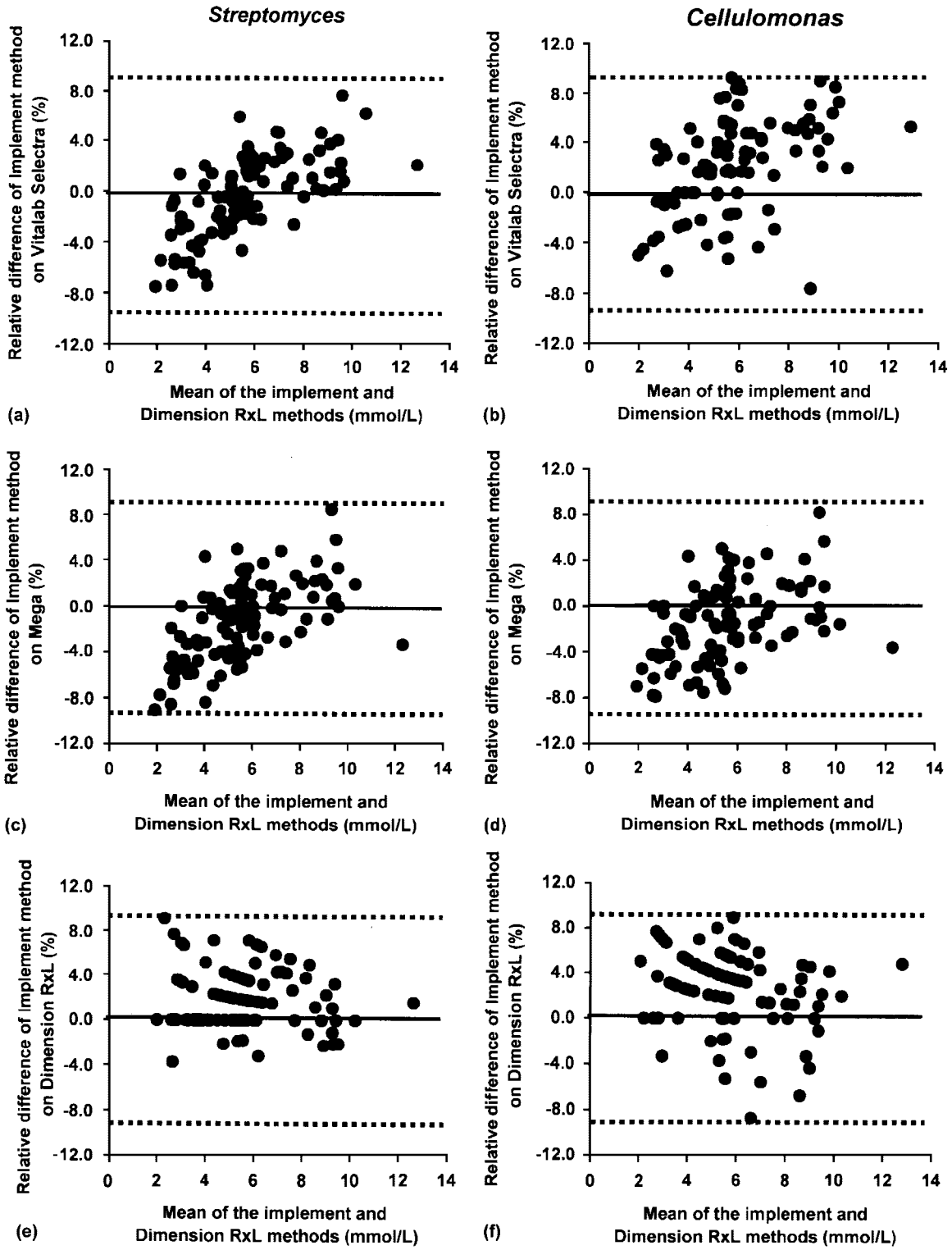

Fig. 2. Different plots of serum cholesterol obtained from relative difference of the present methods using reagents contained cholesterol oxidase from *Streptomyces* (**a**, **c**, **e**) or *Cellulomonas* (**b**, **d**, **f**) on Vitalab Selectra (a and b), Mega (c and d), and Dimension RxL (e and f) analyzers and the commercial Dimension RxL's method vs. the average value of two methods. The *y*-axis is the % relative difference obtained from test method minus Dimension RxL's method, divided by Dimension RxL method and multiplied by 100, and the *x*-axis is the average value of two methods. Dotted lines indicate the acceptable limit (total analytical error for cholesterol as $\pm 9\%$).

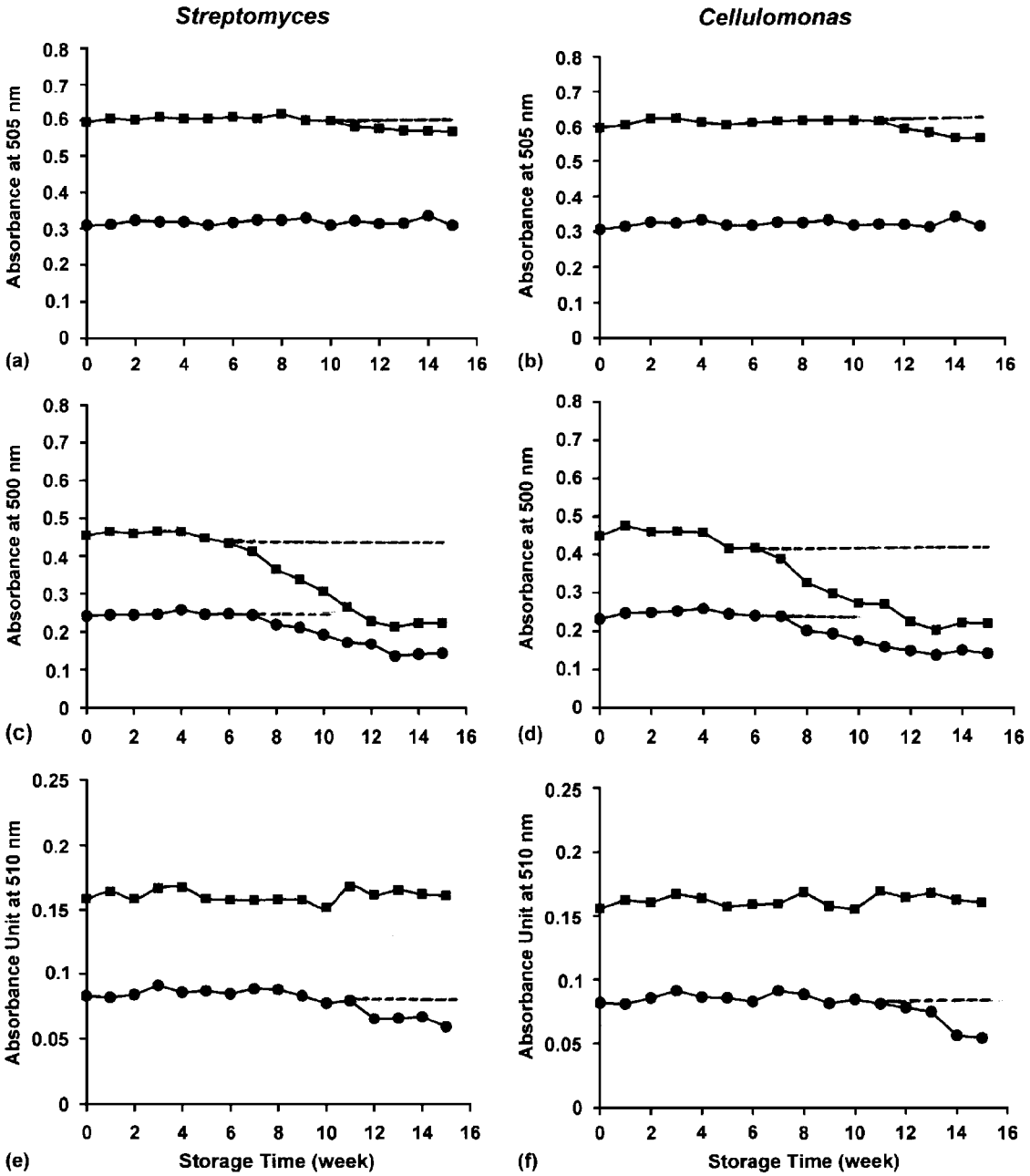

Fig. 3. Stability of cholesterol reagent contained enzyme from *Streptomyces* (**a**, **c**, **e**) or *Cellulomonas* (**b**, **d**, **f**) on Vitalab Selectra (a and b), Mega (c and d), and Dimension RxL (e and f) analyzers kept at 2–8°C. The circle symbol represents normal cholesterol level (4.70 mmol/L). The square symbol represents high cholesterol level (8.81 mmol/L). Dotted lines represent the assumed line of the stable reagent.

those of the other enzymes, which makes it more costly (more than four-fold). The cost and performances of both *Streptomyces* and *Cellulomonas* cholesterol oxidase are quite similar. We introduced *Cellulomonas* as a new source of cholesterol oxidase in addition to *Streptomyces*, for determining serum cholesterol by the enzymatic endpoint method.

To assess the reliability of *Cellulomonas* reagent for cholesterol determination, we evaluated the analytical performances characteristics of this reagent and compared with *Streptomyces* enzyme on various clinical analyzers. The upper end of the reportable range of cholesterol depended on the analyzer being used. There was no difference in the linearity obtained for *Streptomyces* and *Cellulomonas* cholesterol reagents on each instrument.

To achieve reliable classification of patients, NCEP expert laboratory panels (4) have established requisite

analytical performance goals based on clinical needs. The total error (<9%) can be achieved by an imprecision of ≤3%, and a bias compared with the reference method of ≤3%. We found that the patient results obtained from the implemented methods met the requirements for total analytical error goal. The analytical errors of all implemented assays were within the NCEP recommendation (Table 4). No difference in the reproducibility was seen between each reagent and equipment. In addition, the bias criterion for the cholesterol determination from the reference CDC method was met by using both cholesterol reagents. Thus, both enzymes are clinically acceptable.

The results of our studies indicate that a dual wavelength measurement as used on the Mega and Dimension RxL methods (Table 1) can eliminate the absorption of hemoglobin, but a single measurement (Vitalab Selectra) cannot. These data further support the study of Deacon and Dawson (13) that the use of a blank measurement can correct a positive interference of hemoglobin. To eliminate the effect of hemoglobin on the endpoint cholesterol determination, we propose to use the method with bichromatic measurement. The effects of bilirubin and turbid on cholesterol assay are similar to the previous study (7). We observed no difference in the effect of interfering substances between the method using *Streptomyces* and *Cellulomonas* cholesterol reagent.

The stability of *Cellulomonas* cholesterol reagents was similar to the *Streptomyces* cholesterol reagents on each instrument. From our results based on the linearity and the stability, we found that the analytical performance may be dependent on the analyzers being used. Endpoint methods using long incubation periods may be more preferable than those using shorter ones, perhaps because some enzyme activity may have deteriorated due to long reagent storage. Altered enzyme activity minimally affects methods with long incubation times, because the enzymatic reaction will have time to reach completion.

Cellulomonas derived cholesterol oxidase demonstrates a major advantage over that derived from *Streptomyces* in that it does not require shipment on ice (−20°C). Frozen transport is unreliable in many tropical and subtropical countries and even in the southern parts of the United States during summer months (reagents often sit on loading docks for hours before they are delivered to the laboratory), thus *Streptomyces*-derived cholesterol oxidase may not appropriate for many geographic reasons. For this reason, the *Cellulomonas*-derived cholesterol oxidase may represent the best source of enzyme for use in cholesterol determination by the endpoint method.

The performance characteristic of enzyme as evaluated in this study may be modified for lipid and lipoprotein determination. All measurement assays for HDL and LDL basically use the same quantification principles in determining the level of target lipoproteins by using surface-active agents and polyanions (14–17). After lipoprotein selection, each particle measurement requires quantitative of their cholesterol contents. Therefore, the cholesterol reagent using *Cellulomonas* cholesterol oxidase may be developed to the measurement of HDL-C and LDL-C.

CONCLUSION

The excellent performance characteristics of *Cellulomonas* cholesterol oxidase allow its use for serum cholesterol determination by the endpoint method. There was no difference in the analytical performance between *Cellulomonas* and *Streptomyces* enzymes. The method using each enzyme proved the appropriate assay for cholesterol determination used in routine clinical laboratory because its accuracy and precision meet the currently established analytical performance goals. A method comparison study with the CDC standardization method showed excellent correlation and acceptable agreement between results. In addition, we suggest the methodologies using the dual wavelength measurement and long incubation time are preferable for analytical determination by the endpoint technique.

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