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Estimation of Total Cholesterol from A Single Dried Blood Spot - New Application for an Age old Technique!

KEYWORDS: Dried Blood Spot, Cholesterol, Cholesterol Oxidase-peroxidase, Cardio vascular disease, ROC analysis

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ABSTRACT *Dried Blood Spot (DBS) technology is an age old technique used for analysis of various metabolites in screening infants for metabolic disorders. The same has been used to estimate serum Cholesterol levels. Cholesterol is either supplied through diet or produced by the liver. However, an excess of cholesterol intake and minimal physical activity causes the formation of atherosclerotic plaques in the blood vessels leading on to cardiovascular diseases. DBS technique is simple, minimally invasive, cost effective and easy to handle. Hence, it can be used in epidemiologic surveys for screening of Non Communicable Disease risk.*

In this pilot study, the accuracy of the DBS method was assessed against the well-established methods of cholesterol estimation using serum samples. The effect of storing the DBS samples for a week was also determined. The samples were obtained by simple random sampling and the cholesterol levels were estimated using the Cholesterol Oxidase - Peroxidase method. The data was analyzed using Pearson correlation, ROC curve analysis and linear regression analysis. It was concluded that total cholesterol estimated using the dried blood spot sample was an excellent low cost test that can be used as a screening marker for early detection of cardiovascular disease risk.

INTRODUCTION:

Cholesterol is a major marker in measuring cardiovascular disease (CVD) risk. CVD is one of the leading causes of death with hypercholesterolemia being the most important modifiable risk factor in both developed and developing countries [1]. Excess cholesterol in the human body doesn't manifest itself clinically and hence any internal disease scenario is not appreciated by the individual and it goes unnoticed till an episode of myocardial infarction or stroke occurs. In developing countries people are well aware of the risks of hypertension, but the same general awareness for hypercholesterolemia is not seen [1].Thus, it is important to measure cholesterol levels regularly and also conduct epidemiological surveys of the cholesterol status in the society, as the modern lifestyle predisposes the community to obesity, increased blood cholesterol levels and cardiovascular disease. Cholesterol is a sterol, which is present in all the cells in the body and is obtained from two sources. The body synthesizes some cholesterol, and the rest is obtained from the animal food sources such as meat, milk, eggs, or their derivatives. Excess consumption of these types of food might increase the total amount of cholesterol in the body. Cholesterol travels in blood in particles called lipoproteins. Total cholesterol is made up of LDL cholesterol, HDL cholesterol, and VLDL cholesterol. A desirable level of total cholesterol is less than 200 mg/dl. Measurement of cholesterol levels provides the patient and doctor with the necessary data to assess risk for cardiovascular disease and to prevent, as far as possible, a cardiovascular event such as a heart attack or stroke [2].

The use of dried blood spots (DBS) obtained from heel or finger pricks and spotted onto filter paper for the collection and analysis of human blood dates back to the early 1960s when Dr Robert Guthrie used the specimens to measure phenylalanine in new born for the detection of phenylketonuria. This novel approach of blood collection led to the population screening of new born and other clinical testing. Now, DBS is used for the estimation of various metabolites in the blood. Dried Blood Spot (DBS) can be used as a simple, cost effective and accurate tool for the estimation of cholesterol levels in the body. DBS technology is well known, but with this study, a new application is being developed for it. DBS offers a number of advantages over conventional whole blood, plasma or serum sample collection. First, it requires a less invasive sampling method which minimizes the need for a trained phlebotomist, it also requires a small sample volume, which improves patient compliance and will be useful in paediatric studies [3]. Second, since a dried blood spot can be stored at room temperature, refrigeration is not necessary for storage or shipping, which greatly reduces costs. Third, chemically coated dried blood spot cards are less hazardous than plasma tubes, as they offer bacterial lysis. This results in easier transportation, because Dried Blood Spot samples can be shipped as non-hazardous materials using regular mail or courier services [4]. DBS technology can be used for simple, cost effective and reliable testing of metabolites for large scale epidemiologic studies.

DBS samples are now widely being used for measuring serum antibodies and blood hormone levels with good data to define the comparability of results between analyses based upon DBS and standard venous samples. However, there is insufficient data to define the same for cholesterol estimation [5]. Despite all its advantages, DBS analysis of metabolites would not prove useful if they were not accu-

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rate. As there is insufficient data, the need for this study is to assess the reliability of cholesterol values obtained by using a dried blood spot against the standard venous sample cholesterol in our study population.

The use of Dried Blood Spots as an alternative method of sample collection for estimation of cholesterol is a relatively new idea. The absence of well established data to support its reliability and the need for screening of non-communicable disease risk is on the rise in the developing world. There is also an expectation from the medical fraternity for an optimal test that can be made use of to measure cholesterol that will use minimal resources in terms of reagent usage, use of experts in the field to collect DBS or serum samples, procuring of instruments for estimation and at the minimum cost. DBS cholesterol can prove to be a simple yet effective tool to address the global epidemic of cardiovascular disease. A few studies have been carried out in the past few years to establish the reliability of DBS for cholesterol estimation, with varying results. The major findings of the various studies have been summarized here: In a systematic review and meta-analysis of various studies conducted in 2014 [5], 1093 participants were found to provide data to define an association of cholesterol levels measured by various standard methods and compared with DBS cholesterol levels. There was a positive correlation between the DBS cholesterol values and venous blood cholesterol values, but a significant adjustment was required. The linear regression equation obtained was DBS = 0.6807venous + 1.151 (mg/dl). It was concluded that assays based on DBS were clearly associated with assays based on standard venous samples, but there are uncertainties with the significance of this association and further research is required in this field before it can be used as a mainstream epidemiological tool.

In a study conducted at AIIMS, New Delhi, in 2009 [6] under the World Health Organization recommended stepwise approach for Non-Communicable Disease risk factor surveillance in developing countries with minimum resources, 85 blood samples were collected on filter papers circumventing the need for blood processing, storage and shipment at ultralow temperatures. Cholesterol was estimated from the DBS samples and compared to values from venous samples. The correlation coefficient "r" was 0.78 for cholesterol between dried blood spots and serum. The linear regression equation obtained was DBS = 0.727venous + 45.23 (mg/dl). The present study is based on the above study [6], with modifications to further simplify the process and minimize costs. The further simplification of the process in our study included the use of a REMI centrifuge instead of the Environ shaker for elution of cholesterol from the dried blood spot sample. An ELICO colorimeter has been made use of instead of the spectrophotometer or an auto analyzer for taking the absorbance reading from the DBS sample.

MATERIALS AND METHODS

The aim of the present study was to assess the reliability of total cholesterol measurement from a single Dried Blood Spot (DBS) and the objectives of the present study were, to estimate total cholesterol in serum and dried blood spot on the day of sample collection and on day 7 from the date of collection and to assess the accuracy of cholesterol value obtained from DBS sample in comparison with the serum cholesterol. A pilot study was conducted for a period of 2 months by collecting 40 serum and DBS samples by simple random sampling from patients aged between 18 – 75 yrs visiting the M.S Ramaiah Hospital Laboratories. Critically ill patients and patients with known bleeding disorders were excluded from the study.

Rationale for sample size:

Based on a previous study conducted by Dr Lakshmy Ramakrishnan et al. on; Utility of Dried Blood Spots for Measurement of Cholesterol and Triglycerides in a Surveillance Study' [2], it was found that the relationship between dried blood spot and plasma on the day of collection was highly correlated with 'r' value of 0.78. In the present study, expecting similar correlation with a power of 80% and alpha error of 5%, the minimum sample size was estimated to be 37. The sample size will be rounded to 40. Totally, 160 tests will be run using the samples from 40 patients.

On Day 1: 40 serum cholesterol + 40 DBS eluted cholesterol.
On Day 7: 40 serum cholesterol + 40 DBS eluted cholesterol.

Study method:

The study protocol was approved by the institution's ethical committee. After taking the patient's written informed consent, the patient's personal details were recorded and the procedure was explained. A quick general physical examination was performed and the sample was collected and processed as follows.

Discarding the first drop, 5 blood spots were collected on a 1-mm Whatman filter paper after pricking the index finger with a lancet under sterile conditions. This paper was allowed to dry for 2 hours at room temperature and then transferred into a ziploc cover with the patient details and stored at room temperature. A large circular spot of blood was punched out and placed in a test tube with 200 micro liters of methanol. The same was centrifuged at 3000 rpm for 1 hour to elute the cholesterol from the dried blood spot.

About 5 ml of venous blood sample was collected from the patient by standard venipuncture, clotted and then centrifuged to obtain serum sample. Total Blood cholesterol was estimated on COBAS 6000 analyzer by Cholesterol oxidase -peroxidase method on the day of collection. The cholesterol in the serum and from the eluent was estimated by the cholesterol oxidase – peroxidase liquid Kit (EURO Diagnostics, India) method on ELICO CL 157 colorimeter on day of collection and also on day 7. The Enzymatic colorimetric kit was stored at 2-8 ˚C until the expiry date.

Principle of the CHOD – POD method:

Cholesterol esters in serum are hydrolyzed by cholesterol esterase. The free cholesterol produced is oxidized by cholesterol oxidase to form Cholest 4 en- 3-one with simultaneous production of Hydrogen peroxide which oxidatively couples with 4 – aminophenazone and phenol in the presence of peroxidase to yield the red chromophore [7].

50 microliters of the eluent from the DBS was pipetted out into a dry and clean test tube. 50 microliters of serum from the same patient was taken in a separate test tube and 1 milliliter of the enzymatic reagent was added to each of these samples. The test tubes were left undisturbed for 30 minutes and optical density measured at 510nm on an ELICO CL 157 Colorimeter.

Dried Blood Spot standards were obtained by spotting the cholesterol standard provided with the enzymatic kit

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on filter paper and treated similar to the DBS test samples. Standardization was done for both estimation of DBS cholesterol and serum cholesterol using the cholesterol standard provided with the colorimetric enzymatic kit, with appropriate concentrations in each standard. Controls for DBS and serum were prepared and run each time before running the samples to check the reagent stability and reproducibility of the value.

STATISTICAL ANALYSIS AND RESULTS:

The quantitative variables, DBS cholesterol and serum cholesterol were presented using descriptive statistics – range, mean and standard deviation. Correlation was computed between the total cholesterol value obtained from serum and the cholesterol values obtained from Dried Blood Spots using Pearson's correlation coefficient and scatter plots have been plotted to depict the same. Linear regression analysis was used to estimate the total cholesterol based on DBS. To test the equivalence of the two methods, sensitivity and specificity values have been estimated and ROC curves have been plotted. All the data was entered on MS Excel sheet and statistical analysis was carried out using software namely SPSS version 17.0. ROC Curve analysis was done using Med Calc software version 15.8. P value <0.05 was considered as statistically significant.

COMPARISON OF TOTAL CHOLESTEROL LEVELS BY DIFFERENT METHODS

	Reference Ranges for Total Cholesterol	Total Cholesterol by Autoanalyzer CHOD –POD method	Serum Total Cholesterol by enzymatic colorimetric kit method	Total Cholesterol in DBS – enzymatic colorimetric kit method
Group 1	< 200 mg/dl	30	28	31
Group 2	200- 239 mg/dl	07	10	09
Group 3	≥ 240 mg/dl	03	02	00
Total (n)		40	40	40

Table 1: Number of samples within and above normal levels for total cholesterol estimated by different methods

Table 1 shows the number of samples within and above normal levels of total cholesterol in serum and DBS by using Autoanalyzer and enzymatic colorimetric kit method. Group 1 includes samples which have a total cholesterol< 200 mg/dl. Group 2 includes samples which have a total cholesterol level of 200 – 239 mg/dl. Group 3 includes samples which have total cholesterol ≥ 240 mg/dl.

Total Cholesterol (Day 1)	**Range**	**Mean**	**S.D**	
Autoanalyzer cholesterol value (mg/dl)	93 – 264	175.15	40.14	
Serum Cholesterol value by enzymatic colorimetric kit method (mg/dl)	85 – 260	170.27	38.18	
Pearson's correlation between the above two methods				0.966 (p < 0.001)

Table 2: Comparison of total cholesterol levels between auto analyzer and enzymatic colorimetric kit method on Day 1 (S.D. – Standard Deviation) and the Pearson's correlation

Table 2 shows the range, mean and S.D values for the total cholesterol levels obtained on auto analyzer and serum by enzymatic colorimetric kit method on Day 1 and the Pearson's correlation coefficient of 0.966. Figure 1 shows the scatter plot of all values of cholesterol obtained from serum using enzymatic colorimetric kit and auto analyzer on Day 1.

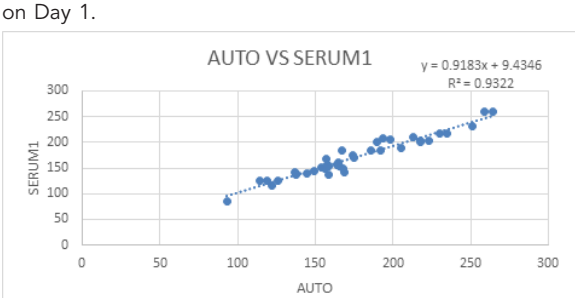

Figure 1: Scatter plot of total cholesterol levels in serum - auto analyser Vs Enzymatic colorimetric kit on Day1

Total Cholesterol (Day 1)	**Range**	**Mean**	**S.D**	
Autoanalyzer cholesterol value (mg/dl)	93 – 264	175.15	40.14	
DBS cholesterol value by enzymatic colorimetric kit method (mg/dl)	75 – 200	145.62	36.60	
Pearson's correlation between the two methods				0.931 (p<0.001)

Table 3: Comparison of total cholesterol levels between auto analyzer and DBS on Day 1 and Pearson's correlation

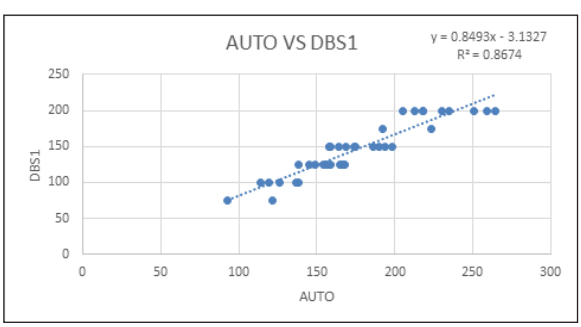

Figure 2: Scatter plot of total cholesterol levels from autoanalyser vs DBS by enzymatic colorimetric kit on Day 1

Table 3 shows the range, mean and S.D values for the total cholesterol levels obtained on auto analyzer and DBS by enzymatic colorimetric kit method on Day 1 and the Pearson's correlation coefficient of 0.931. Figure 2 shows the scatter plot of all values of cholesterol obtained from auto analyzer and DBS by enzymatic colorimetric kit method on Day1.

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Total Cholesterol (Day 1)	Range	Mean	S.D	
DBS by enzymatic colorimetric kit method (mg/dl)	75–200	145.62	36.60	
Serum by enzymatic colorimetric kit method (mg/dl)	85–260	170.27	38.18	
Pearson's correlation between the methods				**0.875 (p< 0.001)**

Table 4: Comparison of total cholesterol levels of DBS and serum by enzymatic colorimetric kit method on Day 1

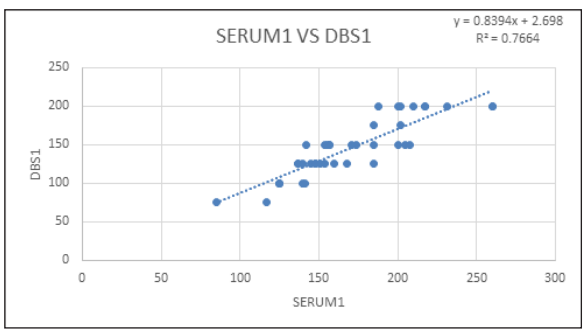

Figure 3: Scatter plot of total cholesterol from dried blood spot and serum by kit method on day 1

Table 4 shows the range, mean and S.D values for the total cholesterol levels obtained on serum by kit method and dried blood spot cholesterol on Day 1 and the Pearson's correlation coefficient of 0.875 between the two methods. Figure 3 shows the scatter plot of all values of cholesterol obtained from serum by kit method and dried blood spot cholesterol.

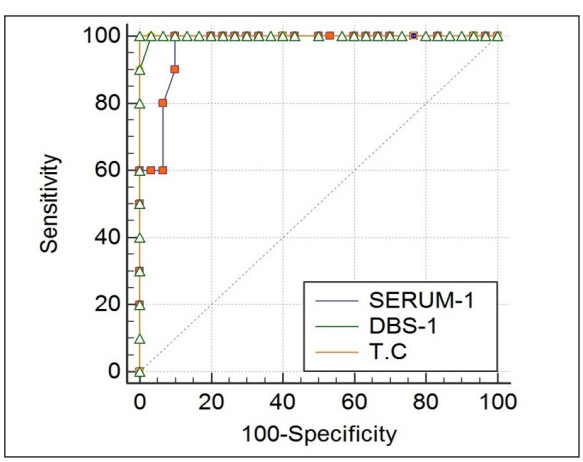

Figure 4: Comparison of ROC curve analysis between the auto analyzer, serum and DBS cholesterol on Day1

Figure 4 shows the Pair wise comparison of ROC curves of total cholesterol levels obtained on the serum in auto analyzer and on serum and DBS by manual enzymatic colorimetric kit method. The Area under the curve (AUC), sensitivity and specificity of the total cholesterol estimated by the auto analyzer and the manual colorimetric kit on serum and DBS sample is depicted in Table 5a and 5b respectively. The AUC for the Serum cholesterol and DBS cholesterol estimated by the manual colorimetric kit method are 0.968 and 0.998 respectively. The sensitivity and specificity for the serum cholesterol by manual enzymatic colorimetric kit method are 60% and 100% as shown in the graph. The sensitivity and specificity for the DBS cholesterol by manual enzymatic colorimetric kit method are 90% and 100% as shown in the graph.

Comparison of ROC curves

Variable 1	T.C (Autoanalyzer)		
Variable 2	SERUM-1		
Variable 3	DBS-1		
Classification variable	TC AA		
Sample size	40		
Positive group ≥ 200 mg/dl	10(25.00%)		
Negative group< 200 mg/dl	30(75.00%)		
Variable	AUC	SE[a]	95% CI[b]
T.C by Autoanalyzer	1.000	0.000	0.912 to 1.000
SERUM-1 by Kit	0.968	0.0239	0.858 to 0.998
DBS-1 by Kit	0.998	0.00348	0.909 to 1.000

Hanley & McNeil, 1982 [8]

Table 5a: Pair wise comparison of ROC curve analysis showing the Area under the curve (AUC)

Total Cholesterol levels	Sensitivity	Specificity
Serum cholesterol by Auto analyzer	100%	100%
Serum cholesterol by Kit method	60%	100%
DBS cholesterol by Kit method	90%	100%

Table 5b: Sensitivity and specificity of Total cholesterol by different methods

ASSESSING STORAGE STABILITY

Table 6 shows the range, mean and S.D values for the total cholesterol levels of dried blood spots on Day 1 and Day 7 and the Pearson's correlation coefficient of 0.955 between the DBS cholesterol on the two different days. Figure 5 shows the scatter plot comparing the values of DBS cholesterol between the two days.

Total Cholesterol	Range	Mean	S.D	
DBS Day 7 (mg/dl)	75 -225	148.12	35.96	
DBS Day 1(mg/dl)	75 -200	145.62	36.60	
Pearson's correlation between two days				0.955 (p<0.001)

Table 6: Comparison of total cholesterol levels between DBS 7 & DBS 1 and the Pearson's correlation

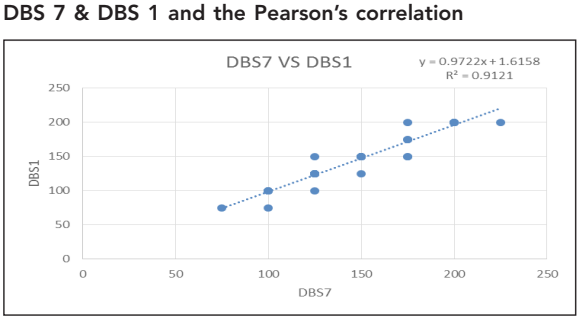

Figure 5: Scatter plot of total cholesterol between DBS Day 1 and DBS Day 7

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	Sample 1 (DBS 1)	Sample 2 (DBS 7)
Sample size	40	40
Arithmetic mean	145.6250	148.1250
95% CI for the mean	133.7677 to 157.4823	136.4769 to 159.7731
Variance	1374.5994	1326.5224
Standard deviation	37.0756	36.4215
Standard error of the mean	5.8622	5.7587
Variance ratio	1.0362	
Significance level	P = 0.912	

Table7: Variance ratio test (F-test) between DBS Day 1 and DBS Day 7

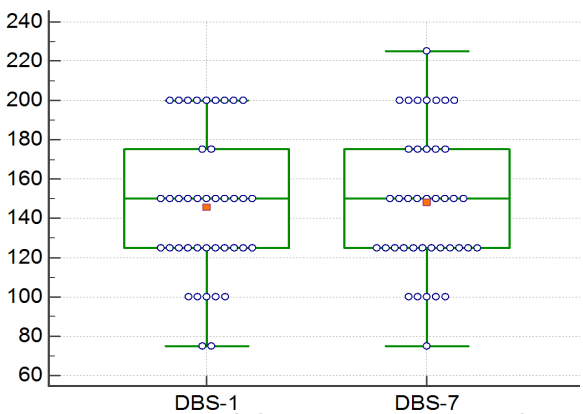

Figure 6: F test graph between DBS Day 1 and DBS Day 7

Table 7 shows the F test or the Variance ratio test. The F statistic or Variance ratio is 1.0362 with a P value of 0.912. Figure 6 shows the graph for F test between DBS Day 1 and DBS Day 7.

DISCUSSION OF RESULTS

Cholesterol is a major marker of underlying cardiovascular disease (CVD) risk. This study involves the estimation and comparison of total cholesterol by enzymatic colorimetric method in serum and DBS sample. The present study was done on a random sample of 40 patients, 21 males and 19 females. According to the NCEP ATP III guidelines, the normal range and risk levels for Total cholesterol are as depicted in Table 8.

Total cholesterol levels (mg/dl)	Risk assessment
< 200	Desirable
200 – 239	Borderline High
≥ 240	High

Table 8: NCEP ATP III guidelines – Total cholesterol levels risk assessment

Total cholesterol level lesser than 200 mg/dl is desirable, 200 – 239 mg/dl is considered as borderline high and anything greater than that is termed hypercholesterolemia. Table 1 shows the number of samples within and above normal levels of total cholesterol in serum by auto analyzer method and enzymatic colorimetric kit method, and also by DBS method. We can infer that almost equal numbers of samples are detected as having hypercholesterolemia by each of the above methods. This indicates that the enzymatic colorimetric kit method using DBS samples are as good as the analyzer in determining hypercholesterolemia. The statistical tests have been applied to determine the correlation between the conventional auto analyzer method and DBS method.

Table 2 depicts the total cholesterol values in serum obtained from the auto analyzer and from the enzymatic colorimetric kit method. By looking at the range, mean and S.D obtained, there seems to be a negligible difference between the two methods used for cholesterol measurement. The Pearson's correlation coefficient for cholesterol between serum and auto analyzer was 0.966, indicating that there is a strong positive linear relationship existing between the two methods. This implies that serum cholesterol obtained by enzymatic colorimetric kit method is as good as the auto analyzer method.

Table 3 shows the range, mean and S.D values for total cholesterol in serum on auto analyzer and DBS sample by enzymatic colorimetric kit on Day 1. The correlation coefficient for cholesterol between dried blood and auto analyzer serum is 0.931, indicating that there is strong positive linear relationship existing between the two methods. A previous study [9] showed a correlation coefficient of 0.797 for the DBS cholesterol estimation. In the previous studies done, the collections of the DBS samples for cholesterol estimation were not standardized and each center made use of the health care workers to collect samples without training them. In the present study the correlation coefficient is better probably because the dried blood spot sample collection was standardized and the sample was collected by the medical student himself.

Table 4 shows the values for the total cholesterol obtained on serum and DBS by using enzymatic colorimetric kit method on Day 1.The Pearson's correlation coefficient was 0.875 indicating the positive linear relationship between the two methods. The linear regression equation obtained in our study was DBS=0.839 venous + 2.698 (mg/dl) which was comparable to a previous study [9] where a linear regression equation of DBS =0.7779 venous + 1.1943 (mg/dl) was obtained. This indicates that there is a need for moderate adjustment of DBS values compared to the standard venous methods.

Figure 4 shows the Pair wise comparison of ROC curves between the serum cholesterol and the DBS cholesterol estimated by enzymatic colorimetric kit method keeping the autoanalyzer cholesterol as the standard method. Table 5a show the Area under the curve for the Serum cholesterol and DBS cholesterol as 0.968 and 0.998 respectively indicating that they are excellent tests to measure cholesterol. The diagnostic accuracy of the ROC curve is based on the following [10]:

Area under the curve	Diagnostic accuracy
0.9 – 1.00	Excellent
0.8 – 0.9	Very Good
0.7 – 0.8	Good
0.6 – 0.7	Sufficient
0.5 – 0.6	Bad
<0.5	Test not useful

Table 9: Diagnostic Accuracy of ROC curve analysis

According to figure 4, the sensitivity of serum cholesterol is only 60% as against the 90% for DBS cholesterol. This is probably due to the presence of interfering substances

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in the serum. A study done previously [11], showed that haemolysis, bilirubin and increased triglyceride levels in the serum showed an interference while determining total cholesterol by end point colorimetric assay in lab instruments using single wavelength (small auto analyzers/ colorimeters/ spectrophotometers) [11]. The same interferences were not seen in instruments using bi-chromatic wavelength as in auto analyzers and hence end point assays were better on auto analyzers. The Kinetic assays for total cholesterol were better in the instruments using single wavelength [11]. The present study made use of the end point colorimetric enzymatic assay which explains the lowered sensitivity. Another study [12] indicates that the Cholesterol oxidase – peroxidase (CHOD-POD) method is subject to interference with various reducing substances such as bilirubin, ascorbic acid and reduced glutathione. In this study a comparison between the methods is being done and hence a sensitivity and specificity of 90% and 100 % for the DBS cholesterol indicates that it picks up almost 90 out of 100 samples which are hypercholesterolemic making it an excellent test in field studies.

The requirement of the blood sample for DBS is only about 0.2 – 0.5 ml which is very minimal as against 2 – 5 ml drawn while measuring serum cholesterol making DBS minimally invasive. In the DBS method, the requirements in terms of lab instruments are only a centrifuge and a colorimeter. The chemical required for elution of cholesterol from filter paper is a simple organic solvent such as methanol.

A comparison of DBS total cholesterol levels were done between day 1 and day 7 to assess the reproducibility of cholesterol values after storing the samples for a week. The DBS samples were stored at room temperature for a week in ziploc bags. The Pearson's correlation coefficient was 0.955 indicating that there was a good correlation between the values even after a week.

Table 7 shows the F test or the Variance ratio test. The F statistic or Variance ratio is 1.0362 with a P value 0.912. The F statistic is the larger variance over the smaller. The P value > 0.05 indicates that there is no significant difference between the values of total cholesterol on day 1 and 7 indicating that DBS stored at room temperature for over a period of one week did not alter the values significantly. This indicates that DBS sample can be stored at room temperature for as long as 7 days for estimation of total cholesterol without affecting the results. A study [13] done previously reported that total cholesterol estimated using DBS were stable for up to 1 month at room temperature, further confirming the findings of our study. Storage of dried blood samples at room temperatures or at 37˚C for up to 60 days did not significantly alter the mean values of cholesterol in another study [14]. However, storage for longer durations for a period of 90 days resulted in 12 per cent reduction in cholesterol levels in the same study [14] and this was attributed to probable bacterial contamination. Our study period was only for two months as the study was a short term student project and hence we committed ourselves to study the storage stability for 7 days only.

A meta-analysis [5] published in the year 2014 denotes that only three studies have been done so far globally to estimate DBS cholesterol. The limitations of the present study include a small sample size of 40, and the highest cholesterol value used in the present study was only 264 mg/dl. The field is open for further studies with increased sample size and including higher cholesterol levels (>264 mg/dl). However the objective of this study to establish DBS cholesterol as a SCREENING TECHNIQUE has been fulfilled.

In this study, we conclude that total cholesterol estimated in the dried blood spot sample is an excellent low cost test and screening marker, for early detection of cardiovascular disease risk which can be used in the primary health care centers or in large scale population studies, especially in developing countries.

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