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Measurement of cholesterol and triglycerides from a dried blood spot in an Indian Council of Medical Research–World Health Organization multicentric survey on risk factors for noncommunicable diseases in India

Ramakrishnan Lakshmy, MSc, PhD*, Prashant Mathur, MBBS, PhD, Ruby Gupta, MSc, PhD, Bela Shah, MBBS, MD, Krishnan Anand, MBBS, MD, Viswanathan Mohan, MD, PhD, DSc, Nimesh G. Desai, MBBS, MD, Jagdish Mahanta, MBBS, MD, Prashant Prabhakar Joshi, MBBS, MD, Kavumpurathu Raman Thankappan, MBBS, MD, MPH

Department of Cardiac Biochemistry, All India Institute of Medical Sciences, New Delhi–29, India (Dr. Lakshmy); Division of Non–Communicable Diseases, Indian Council of Medical Research, New Delhi, India (Dr. Mathur); Department of Cardiac Biochemistry, All India Institute of Medical Sciences, New Delhi, India (Dr. Gupta); Division of Non–Communicable Diseases, Indian Council of Medical Research, New Delhi, India (Dr. Shah); Centre for Community Medicine, All India Institute of Medical Sciences, New Delhi, India (Dr. Anand); Madras Diabetes Research Foundation, Chennai, India (Dr. Mohan); Institute of Human Behavior and Allied Sciences, Delhi, India (Dr. Desai); Regional Medical Research Centre, Dibrugarh, India (Dr. Mahanta); Department of Medicine, Government Medical College, Nagpur, India (Dr. Joshi); and Achutha Menon Centre for Health Science Studies, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, India (Dr. Thankappan)

KEYWORDS:
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Cholesterol;
Coefficient of variation;
Dried blood spots;
Intraclass correlation coefficient;
Triglycerides

BACKGROUND: Dried blood may be a convenient method of sample collection in epidemiological studies; however, the method needs evaluation in a field settings. In the present study, feasibility of using dried blood for measurement of cholesterol and triglycerides was evaluated in multicenter surveillance study for noncommunicable disease (NCD).

METHODS: Samples were collected in a cross-sectional study for NCD risk factor surveillance conducted in six centers in India. For every tenth subject recruited, a blood sample was also collected on filter paper. These 10% serum samples and dried blood spots were analyzed for cholesterol and triglycerides.

RESULTS: The mean coefficient of variation (CV) for cholesterol was less than 10% between dried blood and serum in five of the six participating centers. Only one center showed a high CV of 14%. Similarly, the mean bias was less than 10% in five centers. The intraclass correlation between cholesterol values in dried blood and serum were greater than 0.638 in all centers, which suggests a good homogeneity of results. The mean CV for triglycerides ranged from 0.36% to 17.97%. The intraclass correlation between triglyceride values in dried blood and serum ranged from 0.756 to 0.880 in the six centers.

CONCLUSION: In conclusion, dried blood would be a good method for collection of blood for measurement of cholesterol and triglycerides for population health surveys. However, the benefits of

* Corresponding author.
E-mail address: lakshmy_ram@yahoo.com
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blood spot analysis should be weighed against potential sources of errors attributable to sampling and other factors, such as temperature and humidity, in a country like India.

Coronary artery disease (CAD) is the leading cause of death and a major cause of morbidity worldwide. The disease has been estimated to affect 10% of adults older than 35 years of age in Indian cities and to have caused 1.5 million deaths in India in 2000.[1] Environmental factors that are modifiable as well as genetic factors contribute to the predisposition to CAD. Cholesterol and triglyceride levels have been associated as important modifiable risk factors for CAD.[2,3] LDL cholesterol-lowering has an important role to play in primary and secondary prevention of CAD.[4,5] The importance of triglyceride levels as a risk factor for CAD has been underemphasized until the authors of a recent meta analysis of various epidemiologic studies confirmed the link.[6] The measurement of triglyceride levels is particularly important in Indians, who have a triad of high triglyceride levels along with high low-density lipoprotein (LDL) cholesterol and lower high-density lipoprotein (HDL).[7]

Asian Indians with CAD have similar levels of cholesterol as compared with white subjects but greater triglyceride levels. Triglycerides bring about a change in LDL particle size, density, distribution, and composition, producing smaller, denser, and more atherogenic particles. Both cholesterol and triglycerides, being modifiable risk factors, are amenable to change through public health and clinical interventions and therefore warrant early detection at the individual level and surveillance at the population level. Screening of cholesterol and triglyceride levels in the population would help to identify people at risk for CAD and would aid in formulating preventive strategies. There have been surveillance studies in industrial population across India[8] as well as numerous cross-sectional surveys[9–11] for identification of risk markers for cardiovascular disease.

For Noncommunicable disease risks factor surveillance, including CAD surveillance, The World Health Organization has recommended a STEPwise approach.[12] Step 1 involves collecting information on key risk factors by the use of questionnaires, then moving to simple, physical measurements in the next step (step 2), and only then recommending the collection of blood samples for biochemical assessment (step 3). The biochemical assessment at the population level has been a challenge because of the complexities of measurement involved. In a large country such as India, the biochemical analysis at remote corners of the country is difficult because of limited resources and technical capacity. Measurement in a good quality central laboratory would be ideal but cost, safety, and logistics of the transportation of chilled samples has always been a major concern. The transportation of samples in the form of dried blood would circumvent the need for blood processing, storage, and shipment at ultra-low temperatures. Dried blood would be an attractive option for collection and transportation of samples from remote and inaccessible places. A drop of blood collected by finger prick on filter paper is a relatively noninvasive method for collection and improves participation rate.[13]

The stability of cholesterol and triglycerides in dried blood has been demonstrated previously in laboratory conditions.[14–16] However, before widespread use of dried blood is advocated, studies need to be conducted in the field settings to assess the influence of factors, such as the quality of blood spots, temperature, humidity, and transportation conditions that come into play in a population-based study that can potentially affect the levels of analyte. We have earlier described the use of dried blood for measurement of cholesterol and triglycerides in a surveillance study in which dried blood was collected in field and transported to laboratory located in close proximity for analysis.[17]

The collection of dried blood from field setup in remote corners of the country that vary in temperature and humidity conditions as well as differing in technical capabilities and resources and the effect of transportation to a distant laboratory were not assessed in that study. In the present study, the feasibility of using dried blood for measurement of cholesterol and triglycerides was evaluated in multicenter risk factor surveillance study for noncommunicable disease (NCD), which also collected data on behavioral and physical NCD risk factors.

Methods

A community-based cross-sectional study for NCD risk factor surveillance was conducted in six centers spread across the country viz. Ballabgarh, Chennai, Dibrugarh, Nagpur, Trivandrum, and New Delhi (IHBAS)[18,19] from 2004 to 2005. The study collected information on behavioral risk factors (step 1), ie, consumption of alcohol and tobacco, fruits and vegetable intake, physical activity; and physical risk factors (step 2), ie, weight, height, blood pressure, and waist circumference. These data were collected from one person per selected household, in male and female subjects between 15 and 64 years of age. When the STEPS sample frame was used, at each participating site there were 250 participants in each age (15–24, 25–34, 35–44, 45–54, 55–64 years) and sex category. Thus, the target sample size at Ballabgarh, Chennai, Dibrugarh, Nagpur, and Trivandrum was 7500 subjects in all the age, sex, and population categories. At the Delhi center, the target was 5000 subjects because the center did not include rural areas in its population to be studied.

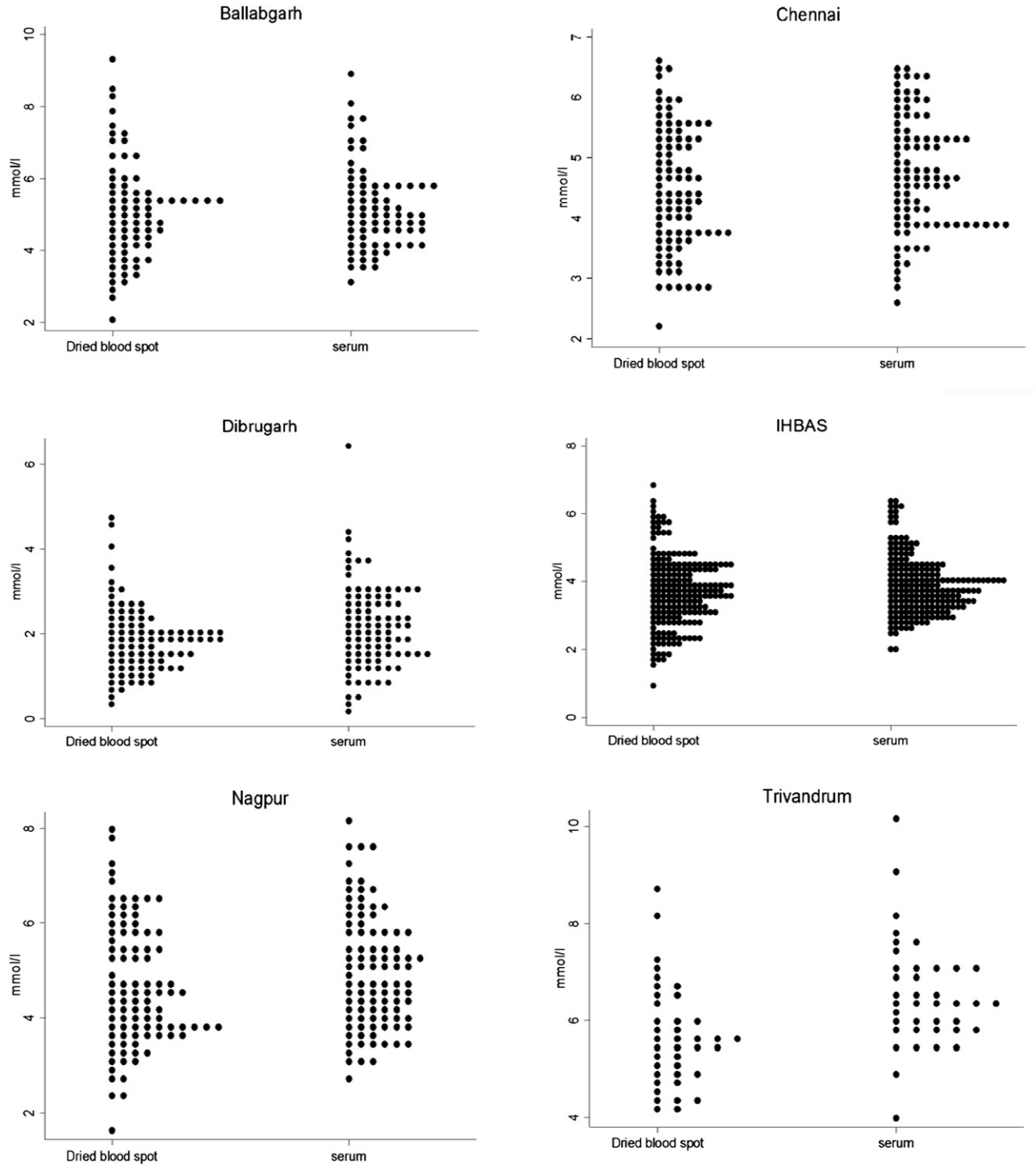

Figure 1 Dot plot of cholesterol values (mmol/L) in dried blood and serum from the six centers.

Overall, at the end of the study, at six sites 42,500 subjects were recruited. The urban areas were recruited by all six sites, whereas rural areas were studied at Ballabgarh, Chennai, Dibrugarh, Nagpur, and Trivandrum and urban slum areas were included at Ballabgarh, Chennai, Delhi, Nagpur, and Trivandrum. At Dibrugarh site, peri-urban population was recruited instead of slums. Biochemical estimation of fasting blood glucose, total cholesterol, HDL cholesterol, and triglycerides was undertaken in the same urban, rural, and slum population where the earlier study with step 1 and 2 was performed in 2003 to 2004. Thus, the study was performed in 20% of the previous sample size studied, and 50 blood samples were collected per age and sex category in three population areas (urban, rural, and slum). For step 3, which involved biochemical measurements, 7483 samples were collected from the six sites in the study. Approximately 10% of samples (748) were collected for repeat analysis. Dried blood was available for 613 of these samples.

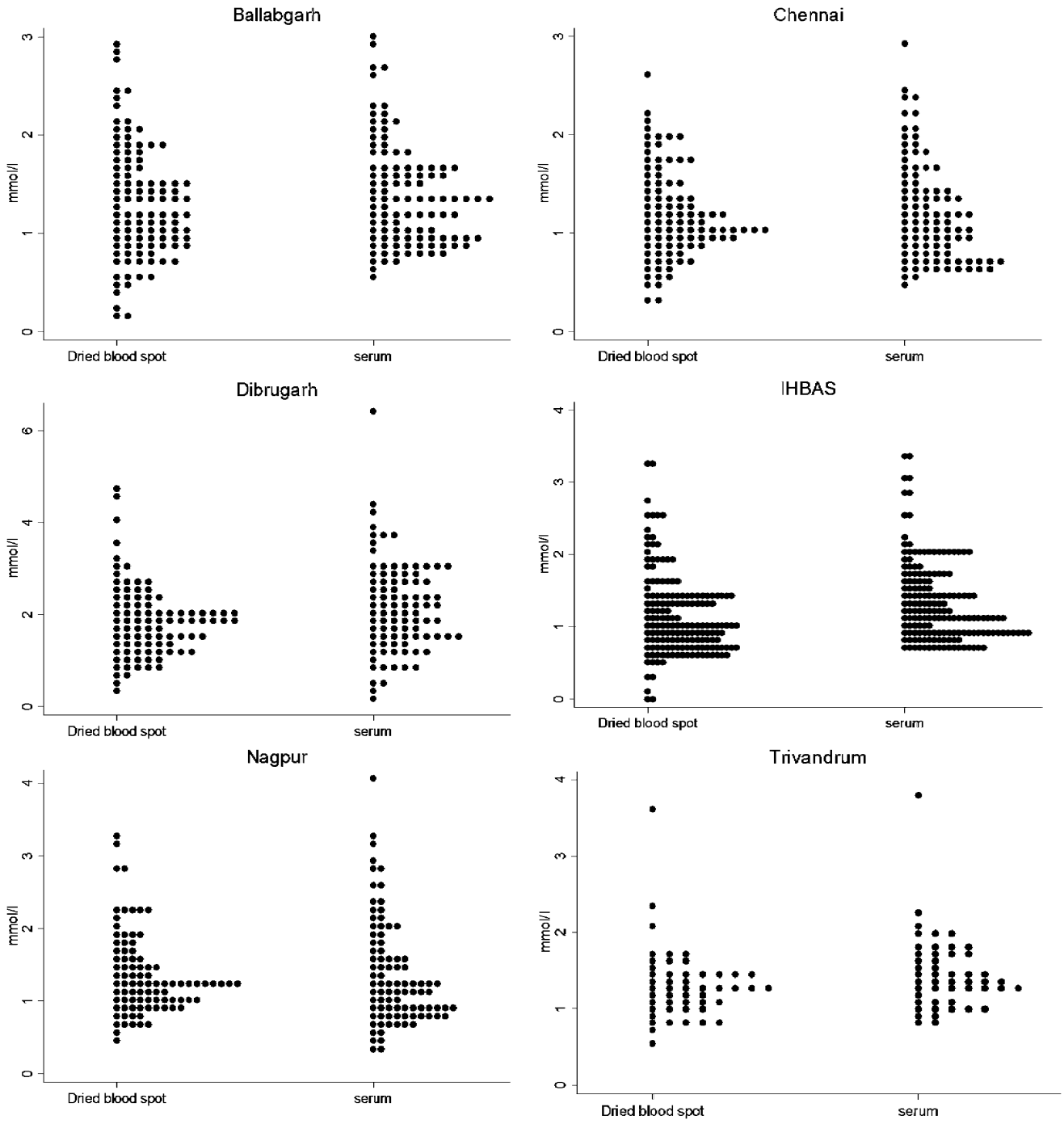

Figure 2 Dot plot of triglyceride values (mmol/L) in dried blood and serum from the six centers.

Because house-to-house collection of blood samples was logistically inconvenient at most times, research teams gave appointments to the selected respondents for reporting at a common place (targeted camp) that was convenient to the participants. Because fasting samples were to be collected, the selected respondents were advised in writing about the instructions and the time in early morning to assemble. To maximize participation and compliance, the appointments also were given on holidays. After ensuring that the subjects were fasted overnight for at least 12 hours, 5 mL of blood was collected via aseptic venipuncture into plain tube without any additive. For every tenth subject recruited for the survey, blood sample was also collected on filter paper at the time of blood collection. Five spots of 10 μL each were prepared via the use of a micropipette, as described previously.[17] The remaining blood sample was centrifuged at 2500 g for 15 min to separate serum.

For the preparation of blood spots Whatman filter paper no.3 was used. The filter discs were kept on a thermocol sheet (nonabsorbant surface) for spot preparation. After the blood was transferred, the filter discs were allowed to dry at room temperature for at least 1 hour. Complete drying was

ensured before transferring the filter discs to resealable bags to protect from dust and moisture. The filter papers with dried blood spots were then kept in refrigerator until they were transported in ice packs to the central laboratory. Cardiac Biochemistry laboratory, located at All India Institute of Medical Sciences (AIIMS), New Delhi, was the central coordinating laboratory for the study.

Cholesterol and triglyceride levels were measured in serum in the local laboratories at each study site as per a uniform protocol. A training workshop was organized by the coordinating laboratory for all the participating laboratories before the start of the study. The technical staff from each center was given hands-on training in all the aspects related to sample collection and analysis. They were all briefed about the internal quality control and external quality assurance practices to be followed during the entire study. The importance of appropriate storage and their transportation were elaborated. Reagent kits for analysis of cholesterol and triglycerides in all the laboratories were centrally purchased by Indian Council of Medical Research after approval of expert committee and sent by the Coordinating Laboratory to each site for maintaining uniformity. The kits used were from RANDOX (Antrim, UK). Cholesterol was estimated by CHOD-PAP method and triglycerides by GPO-PAP method. The same batch of reagents were sent to all laboratories.

For quality assurance, three measures of quality checks was implemented: internal quality control, external quality assurance, and 10% repeat analysis in the coordinating laboratory. The analysis in dried blood and the 10% repeat samples was performed in coordinating laboratory by use of the same batch of reagents as sent to the laboratories in the center. The serum samples for 10% repeats and their dried blood spots were transported to the coordinating laboratory once every month. We are reporting here the comparison between cholesterol and triglycerides estimated in 10% of serum samples and their dried blood spots, both analyzed at the coordinating laboratory.

All the participating institutions had taken approval from their respective ethical committee for the main study as well for the dried blood study. The study was performed as per ethical guidelines laid by the institutions. All the participants provided informed consent.

Lipid measurement in dried blood

For dried blood lipid measurement, one disc was punched out and put in tube with Teflon screw cap. Then, 100 μL of methanol (Analytical grade; Qualigens, Glaxo Limited, India) was added into the tube. The tubes were incubated at 37°C for 2 hours, with shaking at 100 rotations per minutes in an Environ Shaker (Lab-Line Instruments, Inc., Melrose Park IL). For measurement of total cholesterol and triglycerides in the eluate, 20 μL of the extract was taken into microtitre plate and 200 μL of the commercially available enzymatic reagent was added. The reaction mixture was stirred on a vortex mixer with microplate attachment and incubated at 37°C for 15 min and measured at 540 nm on a microplate reader (Rayto Inc., China) via the use of whole blood zero standard as blank. To minimize matrix differences and maximize comparability between calibrators and test samples, dried blood spot standards and controls were prepared by mixing washed red blood cells with the cholesterol and triglycerides standard provided with the enzymatic kit and three levels of quality control. The lipid standard was serially diluted in normal saline, and washed erythrocytes were mixed in proportions 50:50 (v/v) to obtain whole blood calibrators. The blood-based quality controls were similarly prepared by adding washed erythrocytes in 1:1 dilutions.

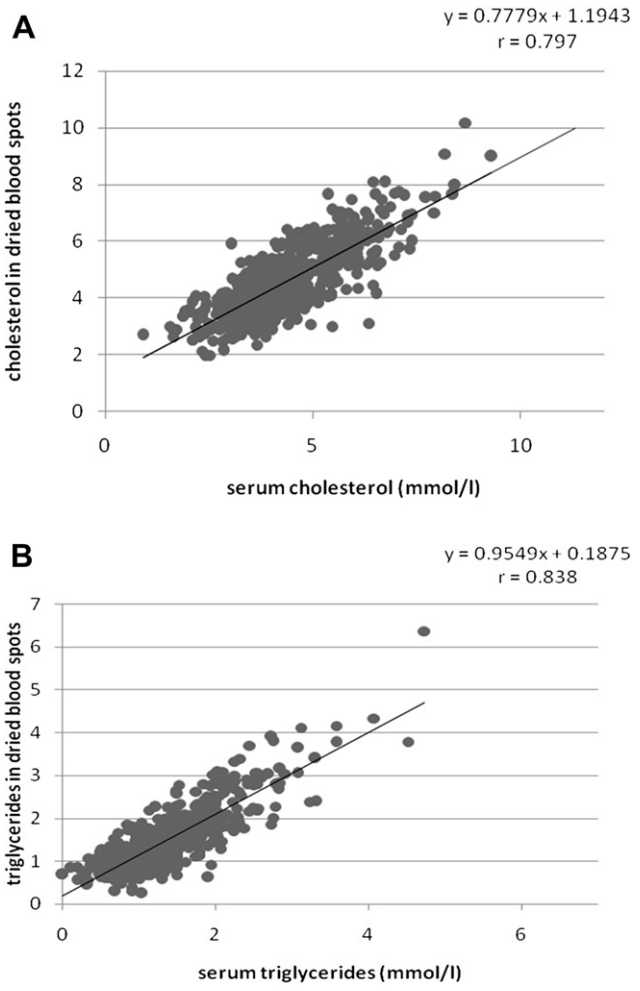

Figure 3 Scatter plots of (A) cholesterol and (B) triglycerides in dried blood spots and serum from all the centers computed together (n = 613).

Statistical analysis

Dot plot and scatter plots were prepared to compare cholesterol and triglyceride values obtained in dried blood as against that obtained in serum. The mean difference between the values obtained from serum and dried blood were compared by calculating intra class correlations. Within-pair CV was computed for the dried blood samples,

Table 1 Analysis of difference in cholesterol and triglyceride values obtained in dried blood and serum in six centers

Centers	Dried blood	Serum	Mean difference	Mean CV, %	Bias, %	ICC
Mean cholesterol, mmol/L						
Ballabgarh	5.07 (1.40)	5.15 (1.16)	0.10 (0.81)	3.53	2.03	0.823
Chennai	4.49 (1.01)	4.74 (0.95)	0.25 (0.62)	5.95	5.26	0.787
Dibrugarh	4.32 (4.79)	4.39 (1.07)	0.07 (0.73)	1.62	1.76	0.769
Trivandrum	5.66 (1.03)	6.51 (1.11)	0.85 (0.58)	14.10	13.06	0.851
Nagpur	4.64 (1.28)	4.95 (1.20)	0.31 (1.07)	7.50	6.31	0.638
IHBAS	3.71 (1.04)	3.88 (0.86)	0.17 (0.71)	6.32	4.14	0.709
Mean triglycerides, mmol/L						
Ballabgarh	1.31 (0.57)	1.38 (0.52)	0.08 (0.34)	9.60	5.63	0.800
Chennai	1.18 (0.45)	1.20 (0.51)	0.015 (0.23)	0.36	1.30	0.880
Dibrugarh	1.87 (0.79)	2.16 (0.98)	0.29 (0.54)	11.60	13.68	0.819
Trivandrum	1.31 (0.47)	1.45 (0.48)	0.14 (0.32)	11.25	10.07	0.776
Nagpur	1.37 (0.56)	1.37 (0.73)	0.006 (0.45)	4.71	0.48	0.756
IHBAS	1.16 (0.55)	1.33 (0.55)	0.19 (0.33)	17.97	13.18	0.782

CV, coefficient of variation; ICC, intraclass correlation; IHBAS, Institute of Human Behaviour & Allied Sciences.
Values are mean and SD in parenthesis.

and bias between dried blood spots and serum levels of cholesterol and triglycerides was calculated by considering values for serum as true value.

Results

The study was conducted from February 2005 to January 2006 at the six sites via a common protocol. Figure 1 depicts the dot plot of cholesterol values obtained in serum and dried blood in the different centers. A median cholesterol value of 4.97 mmol/L was observed in dried blood as compared with 4.99 mmol/L in serum samples collected from Ballabgarh. In Chennai, the median value obtained with dried blood was 4.37 mmol/L versus 4.66 mmol/L in serum; in Dibrugarh it was 4.14 mmol/L and 4.34 mmol/L; in Nagpur the median values were 4.48 mmol/L in dried blood and 4.76 mmol/L in serum; in Trivandrum it was 5.59 mmol/L and 6.42 mmol/L; and in samples from IHBAS it was 3.70 mmol/L and 3.75 mmol/L, respectively, for dried blood and serum. Distribution of cholesterol values in the different centers (Fig. 1) suggest an underestimation of cholesterol from dried blood as compared with serum in Chennai, Nagpur, and Trivandrum center. The distribution of cholesterol values obtained from dried blood and serum were fairly close in other three centers.

Figure 2 shows the distribution of triglyceride values in dried blood and serum from the different centers. The median values obtained with dried blood were comparable with that of serum with values of 1.22 mmol/L and 1.33 mmol/L, respectively, for dried blood and serum from Ballabgarh; 1.090 and 1.096 mmol/L from Chennai; 1.85 and 2.08 mmol/: from Dibrugarh; 1.24 mmol/L and 1.17 mmol/L from Nagpur; 1.23 mmol/L and 1.32 mmol/L from Trivandrum; and 1.00 mmol/L and 1.19 mmol/L from IHBAS. The spread of triglyceride values were comparable between dried blood and serum in the six centers.

Scatter plot of cholesterol values obtained from dried blood and serum in all the centers computed together is depicted in Figure 3A. An overall correlation coefficient (r) of 0.797 was observed. Figure 3B depicts the scatter of triglyceride values obtained in with dried blood in comparison with that obtained in serum when data from all centers were combined. An overall correlation coefficient of 0.838 was observed.

Figures 4 and 5 give the Bland Altman plots of differences in cholesterol and triglyceride values respectively, obtained with dried blood as compared to serum in the six centers. A good agreement was evident in five of the six centers for cholesterol and all centers for triglycerides. Table 1 gives the analysis of differences in the results obtained with dried blood and serum for cholesterol and triglycerides in the centers. The mean cholesterol differed by 0.07 to 0.85 mmol/L in the different centers with a mean CV of less than 10% between dried blood and serum in five of the six participating centers. Only one center showed a high CV of 14%. Similarly, the mean bias was less than 10% in five centers. The intraclass correlation coefficients between cholesterol values in dried blood and serum were greater than 0.638 in all centers, suggesting a good homogeneity of results. For triglyceride, the mean differences ranged from 0.006 to 0.29 mmol/L, with mean CV ranging from 0.36% to 17.97%. The intraclass correlation coefficients between triglyceride values in dried blood and serum ranged from 0.756 to 0.880 in the six centers.

Discussion

The present study reports the analysis of cholesterol and triglyceride from dried blood collected in the field in a multicenteric NCD risk factor surveillance study coordinated by Indian Council of Medical Research. A reasonably good correlation was observed between values obtained from dried blood as compared with serum for both cholesterol and triglycerides. Mean CV and bias, which is a measure of precision in replicate analysis, was less than 10% in five of the six centers. There were center specific

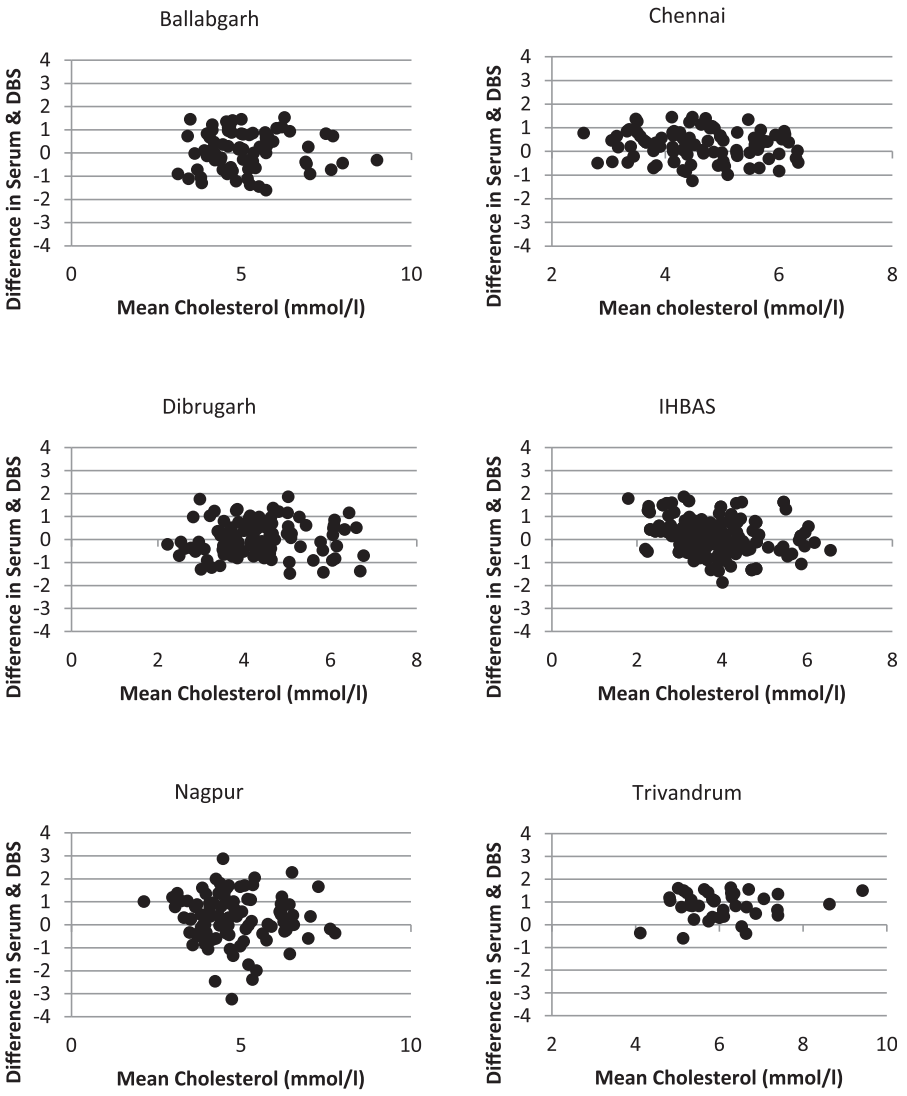

Figure 4 Bland Altman plots of differences between cholesterol values in dried blood and serum.

differences, with overall five of six centers showing comparable results. The use of dried blood in population surveys have been reported previously for neonatal screening for inborn errors of metabolism[20–22] and viral and bacterial disease screening.[23–25] Use of dried blood for measurement of cholesterol and triglyceride in population surveys have not been reported previously. In our previous study we reported the use of dried blood for the measurement of cholesterol and triglycerides in a surveillance study with the collection center in close proximity to the coordinating laboratory; therefore, the issue of collection of blood on filter paper under field conditions and its transportation were not addressed.[17]

Parallel analysis of dried blood and serum with excellent correlation (>0.900) between the two measurements of cholesterol and triglycerides have been reported before under controlled laboratory conditions with trained personnel collecting samples and performing the analysis.[15,16,26] Lesser correlation and greater coefficient of variation observed in the present study could be attributed to suboptimal quality of the blood spots collected in the field as well their improper storage and transportation. The spots were too small in some instances, and discs could not be punched out for extraction. Blood was overlaid on a previously collected spot in some samples. Filters were not dried properly before putting inside the resealable bags. The bags were not closed tightly; as a result, moisture entered inside. Bacterial contamination in such samples can affect lipid levels. The filter papers with dried blood spots were in some instances stored for 6 months before analysis. The stability of dried blood for lipid measurements has only been evaluated for 3 months in the previous studies.[16,17] Also on few occasions the filter discs were received by the coordinating laboratory at ambient temperatures which can influence the lipid levels.

In the field settings with lesser-trained personnel, there can be a number of potential sources of error in blood collection on filter paper, which includes blotting or smearing of blood on paper, overlaying of blood on a

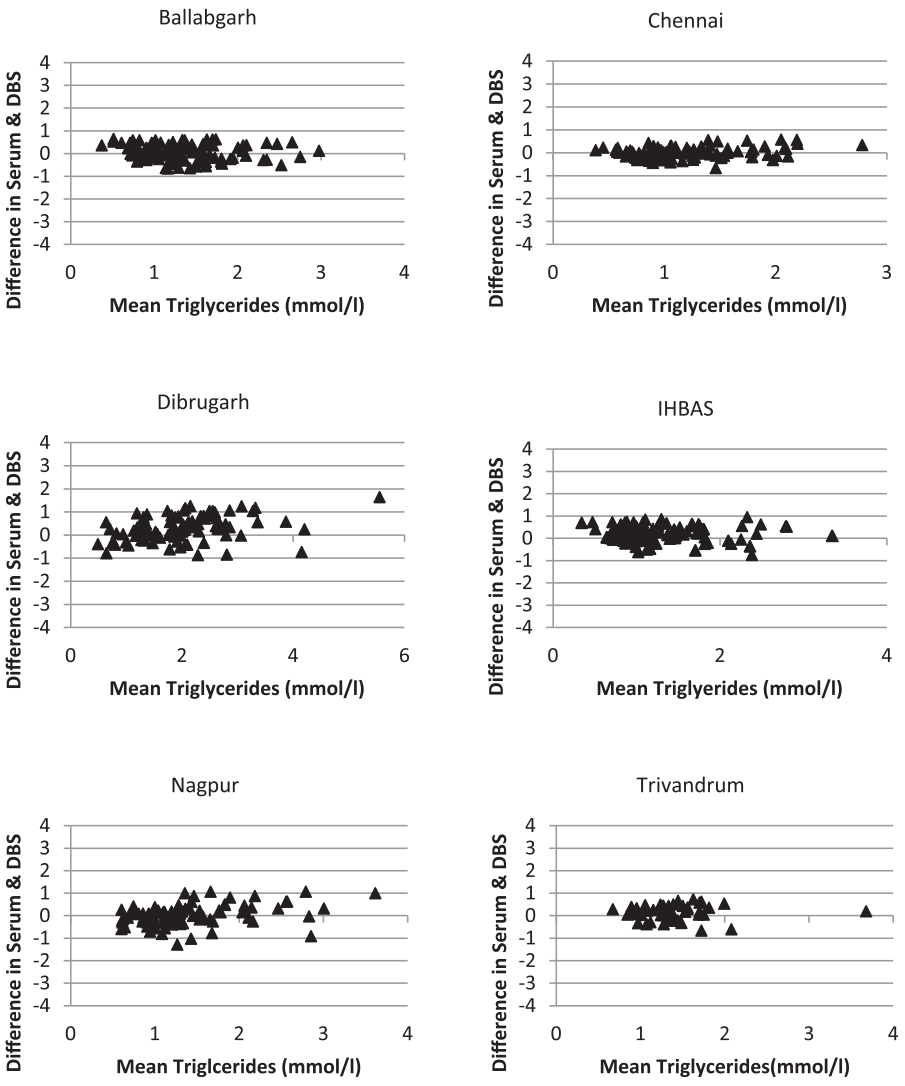

Figure 5 Bland Altman plots of differences in triglyceride values between dried blood and serum.

previously collected spot, and contact between filter paper and blood drop, resulting in improper spot formation and variation in blood spot size.[27] After spotting blood on filter paper, it is important that the blood is allowed to dry properly. Appropriate storage of dried filter disc in resealable bags to prevent moisture is also an important aspect.

The problems faced in the field settings in our study are very similar to the limitations discussed by others.[20,27] The procedures for sample collection need to be standardized; staff personnel require special training on collection technique before the commencement of any program. The central laboratory identified for the analysis of dried blood need to validate the procedures for accuracy, precision, reliability and limits of detection of the analytes of interest. The values obtained from dried blood may not be same as those from serum or whole blood. This may further require development, standardization and identification of a correction factor which can be method and population specific. The technical demand in analyzing dried blood spots are as high as analyses from serum / plasma.

Conclusion

In conclusion, although dried blood would offer an excellent method for collection of blood for measurement of cholesterol and triglycerides for population surveys, the benefits of blood spot analysis should be weighed against potential sources of errors like improper sampling due to limited technical expertise in the field settings.

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