PILOT STUDY

Comparison of dried blood spots with conventional blood sampling for diagnosis of hepatitis b & c through serological and molecular technique; a pilot study

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Abstract

Objective: To compare the sensitivity and specificity of dried blood spot with conventional blood sampling for serological and molecular testing of hepatitis B and C viruses.

Method: The case-control study was conducted at the Pakistan Health Research Council Specialised Centre for Gastroenterology and Hepatology, Karachi, from May,2015 to April,2016 and comprised patients who were hepatitis B surface antigen-positive (group 1), anti-hepatitis C virus-positive (group 2), hepatitis B virus deoxyribonucleic acid-positive (group 3), and hepatitis C virus ribonucleic acid-positive (group 4). A group of controls had healthy subjects negative for both hepatitis B and C viruses. Blood samples were collected using the conventional as well as the dried blood spot method using finger prick. Relevant tests were run for each subject using both the samples at baseline and after 3 and 6 months of storage. Receiver operative characteristic curve was plotted to determine the ideal cut-off points for dried blood spot testing and corresponding sensitivity and specificity. Data was analysed using SPSS 19.

Results: Of the 100 subjects, there were 20(20%) in each of the four patient groups and 20(20%) in the control group. Sensitivity of dried blood spot method was 95.2%, 95%, 80% and 70% for groups 2, 1, 4 and 3 respectively when tested within a week of sampling. Specificity was 100% for all the four groups. There was a significant correlation of the two methods for all the four parameters tested ($p<0.01$).

Conclusion: Dried blood spot sampling correlated well with the conventional blood sampling method for serological and molecular testing.

Keywords: Hepatitis, Diagnosis, Serology, PCR, Dried blood spot. (JPMA 70: 1214; 2020)

DOI: https://doi.org/10.5455/JPMA.23293

Introduction

According to the National Survey on the Prevalence of Hepatitis B and C, Pakistan has more than 13 million cases of hepatitis B virus (HBV) and hepatitis C virus (HCV), making it a country with the second biggest such burden in the world.[1,2] World health Organisation (WHO) 2030 goals are to eliminate Hepatitis B and C from the globe. All countries have to set their diagnosis and treatment targets to meet these goals. In a developing country like Pakistan, we need to have an affordable and accessible diagnostic strategy so that maximum number of patients are diagnosed and linked with care and treatment. Accessibility and affordability is hindered in remote locations as diagnostic tests, like enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), are mostly available in tertiary care hospitals or in private settings. A sampling methodology like dry blood spot (DBS) may not only offer a cost-effective solution to this problem, but is also shown to have several advantages over the conventional sampling.[3]

The concept of DBS sampling was first introduced by Scottish researchers in 1963 for screening neonatal metabolic disease.[4] Since then the use of DBS sampling methodology has been reported in a number of serological, molecular and biochemical assays.[3,5-7]

DBS methodology has several advantages over conventional technique.[8,9] First of all, it is simple to perform and does not require rigorous phlebotomy training as required for venous sampling. Also, handling or processing of specimens to separate serum or plasma from whole blood is not required. Likewise, transportation-related risks, like leakage / breakage of container, are also minimised and there is no need of dry ice packs for transportation of DBS.

Besides, DBS sampling also minimises infection control hazards associated with the use of disposable needles and syringes or with disposal of blood/plasma/serum samples. Refusals in epidemiological surveys associated with the fear of blood sampling can be reduced since capillary blood collection is comparatively less invasive. All these advantages make a huge difference in the cost of

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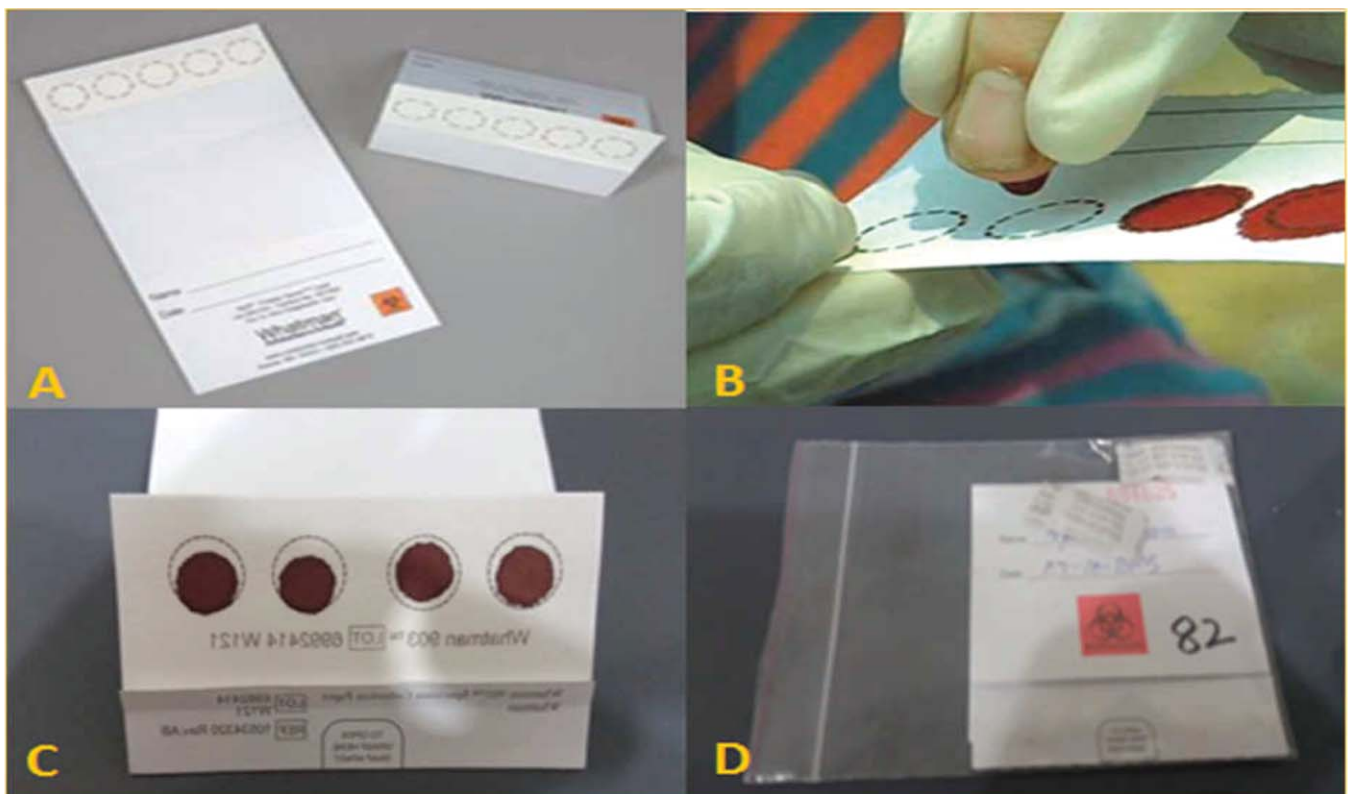
epidemiological studies.[8,9]

as such, DBS sampling may provide a cost-effective, safer and less cumbersome alternative for epidemiological surveys in resource-limited settings like Pakistan. However, it is imperative to establish the suitability of this technique locally as many factors, including hot and humid environment, technical expertise, and storage conditions, may have an effect on the results. The current study was planned to evaluate the suitability and applicability of DBS method for screening of HBV and HCV in Pakistan.

Materials and Methods

The case-control study was conducted at the Pakistan Health Research Council (PHRC) Specialised Centre for Gastroenterology and Hepatology, Karachi, from May, 2015 to April, 2016 and comprised individuals visiting the facility for getting their blood tested for HBV or HCV who were subsequently found to be positive. After obtaining approval from the ethics review committee of Jinnah Postgraduate Medical Centre (JPMC), Karachi, the samples were collected from patients who were hepatitis B surface antigen (HBsAg)-positive (group 1), anti-HCV-positive (group 2), HBV deoxyribonucleic acid (DNA)-positive (group 3), and HCV ribonucleic acid (RNA)-positive (group 4). A group of controls comprised healthy subjects negative for both HBV and HCV. Blood sample of each participant was collected by both conventional venous sampling and fingerprick DBS methods. DBS samples were collected and stored as per the guidelines of the Centers for Disease Control and Prevention (CDC).[10] The sample was collected on filter paper cards (Whatman protein saver cards) using the fingerprick method. These cards were packed in a self-sealing plastic bag (zip-locked) with two silica gel desiccants (0.5g each) to prevent moisture (Figure-1). These packs were then kept in an airtight box and stored at -20°C till further use.

WHO guidelines were followed for the elution of DBS samples.[11] Elution was carried out in Phosphate buffer saline (Oxoid, UK) with mean potential of hydrogen (pH) 7.3±2. A hole punch of 6.35mm diameter was used which was disinfected using 70% isopropyl alcohol (IPA) after every use to avoid contamination. DBS samples were put in a 2ml plasic tube containing 300ul of phosphate-



DBS: dried blood spot.

Figure-1: DBS sampling technique. A) WhatmanTM protein saver filter paper card B) Sample collection C) Air drying DBS samples D) packaging of DBS sample in zipped lock bag with desiccant.

buffered saline (PBS) in case of HBsAg, HBV DNA and HCV RNA, while 100ul PBS was used for anti-HCV testing.

ELISA tests were run on serum samples and the eluted DBS samples simultaneously using diagnostic kits (Murex, Abbott) for HBsAg (3.0) and anti-HCV (4.0). Real-time PCR for HCV RNA and HBV DNA was performed using Qiagen kits for extraction (QIAmp DSP virus Spin Kit) and amplification (Artus HCV RG RT-PCR or Artus HBV RG RT-PCR kits) RNA of viral nucleic acid.

Data was analysed using SPSS 19. Correlation of results by DBS and conventional sampling was determined using Pearson's correlation coefficient. All results were compared with the on respective manufacturer-recommended cut-off values for serum or plasma samples. $P \leq 0.05$ was considered significant. Receiver operating characteristics (ROC) curve analysis was performed to determine the optimal cut-off points for each assay using DBS. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of DBS for each test were determined using the predictive value model of Galen and Gambio [12].

Results

Of the 100 subjects, there were 20(20%) in each of the four patient groups and 20(20%) in the control group (Table-1).

There was an excellent correlation of DBS samples with serum samples for both HBsAg and anti-HCV tests (r=0.93 and r=0.95 respectively). A significant correlation was also observed in case of HCV RNA and HBV DNA (Table-2).

As per ROC analysis, DBS method had sensitivity of 95.2%, 95%, 80% and 70% for groups 2, 1, 4 and 3 respectively (Table-3).

HBV and anti-HCV tests showed no or little effect of storage duration on DBS sensitivity, but HCV RNA showed

Table-2: Characteristics of dried blood spot (DBS) sampling technique for serological and molecular tests of Hepatitis B and C using manufacturer-recommended cut-off points for serum samples.

Characteristics	HBsAg	HBV DNA	antiHCV	HCV RNA
Correlation	0.93	0.95	0.73	0.78
Sensitivity	95.0%	65.0%	70.0%	80.0%
Specificity	100%	100%	100%	100%
PPV	100%	100%	100%	100%
NPV	95.2%	74.1	76.9%	83.3%

HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; DNA: Deoxyribonucleic acid; HCV: Hepatitis C virus; RNA: Ribonucleic acid; NPV: Negative predictive value; PPV: Positive predictive value.

Table-3: Characteristics of dried blood spot (DBS) sampling technique for serological and molecular tests of Hepatitis B and C using receiver operating characteristic (ROC) curve analysis.

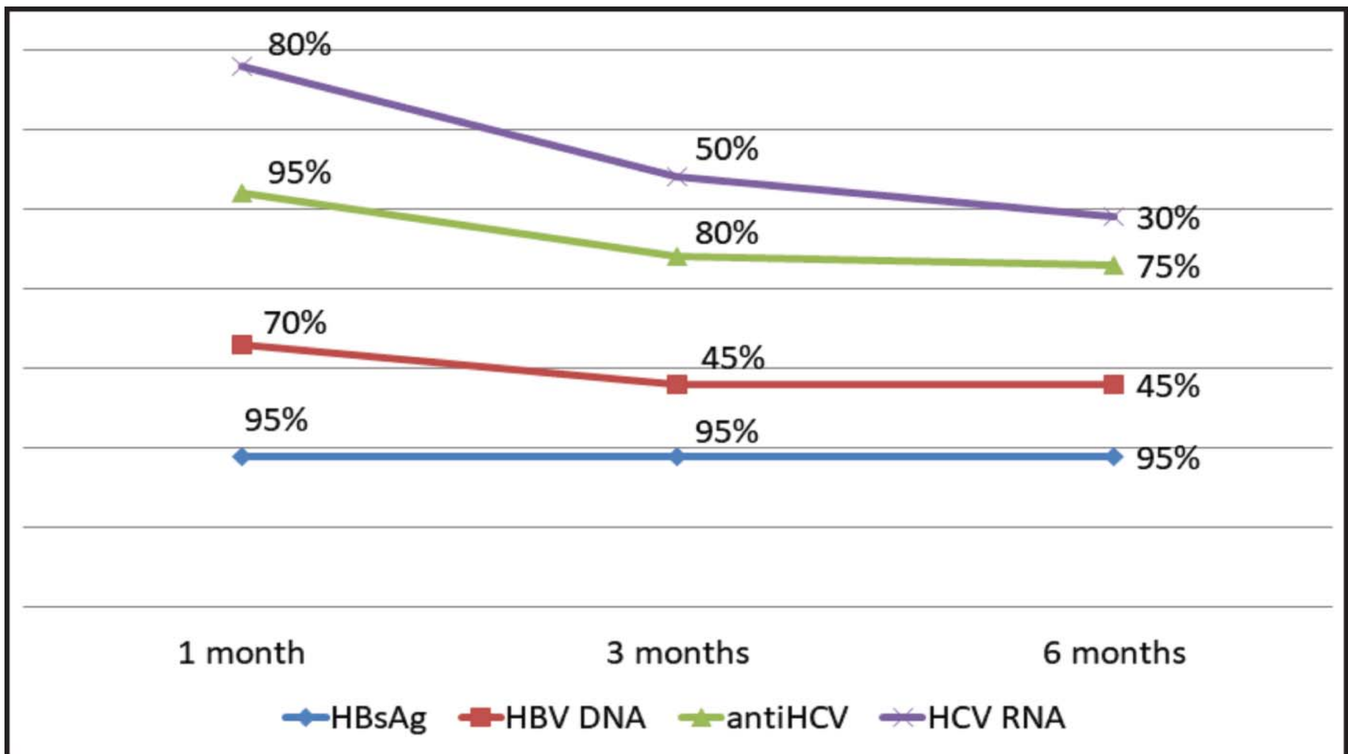
Characteristics	HBsAg	HBV DNA	antiHCV	HCV RNA
AUC	1.000	0.850	1.000	0.900
Pearson r	0.933	0.95	0.73	0.78
Cut-off value	0.312	11.96	0.208	562.65
Sensitivity	95.0%	70.0%	95.2%	80.0%
Specificity	100%	100%	100%	100%
PPV	100%	100%	100%	100%
NPV	95.2%	74.1	95.2%	83.3%

HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; DNA: Deoxyribonucleic acid; HCV: Hepatitis C virus; RNA: Ribonucleic acid; AUC: Area under the curve; NPV: Negative predictive value; PPV: Positive predictive value.

Table-1: Results of serum and dried blood spot (DBS) samples for serological and molecular tests of Hepatitis B and C using manufacturer's recommended cut-off values for serum.

Sample	Test	Result	Serum Samples Positive	Serum Samples Negative	Total
Dried Blood Spots (DBS)	HBsAg (ELISA)	Positive	19	0	19
		Negative	1	20	21
		Total	20	20	40
	HBV DNA (PCR)	Positive	13	0	13
		Negative	7	20	27
		Total	20	20	40
	Anti HCV (ELISA)	Positive	14	0	14
		Negative	6	20	26
		Total	20	20	40
	HCV RNA (PCR)	Positive	16	0	16
		Negative	4	20	24
		Total	20	20	40

HBsAg: Hepatitis B surface antigen; ELISA: Enzyme-linked immunosorbent assay; HBV: Hepatitis B virus; PCR: Polymerase chain reaction; HCV: Hepatitis C virus; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid.



DBS: Ddried blood spot; ROC: Receiver operating characteristic; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; DNA: Deoxyribonucleic acid; HCV: Hepatitis C virus; RNA: Ribonucleic acid.

Figure-2: Effect of storage for different time lengths on sensitivity DBS samples using ROC curve analysis.

a decline at 3 months which further declined at 6 months (Figure-2). Specificity of all the four tests remained unaffected at 3 and 6 months of storage.

Discussion

In the present study DBS sampling showed comparable results with conventional sampling for serological and molecular testing of HBV and HCV. Similar positive observations have been recorded by other studies.[3-5]

Using the cut-off values recommended by kit manufacturers for serum samples, the highest sensitivity (95%) of DBS sampling was recorded for HBsAg testing while the sensitivity was lowest (65%) in case of HBV DNA in the current study. Specificity of DBS samplings was 100% for all the parameters tested.

For HCV testing the sensitivity of ELISA and PCR was 70% and 80% respectively, while specificity was 100% for both tests. Almost similar results have been reported by a recent study; 70.1% and 89.6% respectively.[13]

For Hepatitis B testing, the sensitivity of ELISA and PCR was 95% and 65% respectively, while specificity was 100% for both. A study in Brazil reported 97.6% and 96.7% sensitivity and specificity respectively for HBsAg.[14] Other studies have reported DBS sensitivity for HBsAg as 98% and 96.5% respectively with 100% specificity.[6,15] These results are in concordance with our results, suggesting an excellent correlation of DBS and serum sampling for HBsAg ELISA. For HBV DNA, our study does not suggest a good sensitivity correlation, while another has reported 98% sensitivity for HBV DNA.[15] However, it is noteworthy that the other study used a threshold value of 914.1 IU/ml which was <3.6 IU/ml in the current study. Based on the cut-off used by the other study,[15] the sensitivity of DBS sampling in our sample would increase to 75% which would still be lower compared to the earlier findings,[15] but will be in the acceptable range.

ROC curve suggested 95% and 95.2% sensitivity of DBS for HBsAg and anti-HCV with 100% specificity for both tests at an ideal cut-off point of 0.312RLUs for HBsAg and 0.208RLUs for anti-HCV. The area under the curve (AUC) for both tests suggested a high accuracy of DBS samples compared to the serum samples. Pearson r values also suggested excellent correlation of DBS samples with

serum samples for the two tests. These results are consistent with a previous study conducted in Malaysia.[6]

For molecular testing of Hepatitis B and C, ROC curve suggested high accuracy for HBV DNA and HCV RNA, and sensitivity for HBV DNA and HCV RNA at an ideal cut-off point of 11.96 IU/ml for HBV DNA and 562.65IU/ml for HCV RNA. Pearson r values for both tests also showed significant correlation with serum samples. Studies from other parts of the world also suggest high accuracy and correlation of DBS samples for molecular testing of Hepatitis B and C.[6]

Impact of DBS storage for 3 and 6 months at -20°C was studied on sensitivity and specificity of all four parameters and it showed a gradual drop in the sensitivity of the tests without affecting specificity. While we could not get much data on the topic, but some researchers have reported this effect. One study reported that HCV RNA was stable for up to 10 weeks (i.e. 2.5 months) at room temperature.[16] Another study reported the effect of DBS storage for 7 days at 4°C and 25°C on HBV DNA which did not suggest a significant difference in the viral load of HBV.[5] Both these studies suggested a drop in the positivity ratio and viral load but that was not statistically significant. However, it is noteworthy that storage times in both these studies were lower compared to the current study which found no effect of storage time up to 6 months on HBsAg results, while sensitivity of anti-HCV dropped gradually to 80% and 75% after 3 and 6 months respectively. Molecular tests HBV DNA and HCV RNA showed a noteworthy effect of storage on DBS sensitivity which was most prominent in case of HCV RNA. This might be explained because of the fact that RNA is usually fragile and is easily degraded compared to proteins and DNA. However, we suggest that this effect shall be minimised by carrying out elution of the DBS samples and storing elutes instead of DBS samples if long-term storage is required before testing. Negative effect of storage on HBV DNA and HCV RNA results has been reported serum samples in a study conducted in Australia in which there was a significant drop in HCV RNA and HBV DNA titers of serum samples stored at -20°C and -70°C.[17] However, no study could be found focussing on the effect of storage on sensitivity of serum samples for HBV DNA and HCV RNA.

The present study showed a good correlation of DBS samples with serum for serological and molecular testing of hepatitis B and C. These findings suggest the utility of this technique, particularly for epidemiological studies, where many issues involved in blood sampling for serum, like cost, need of technical training, related infection control hazards, transportation hazards etc., can be avoided. Also, DBS technique may be useful for routine testing of HBV and HCV in children in whom blood sampling is difficult or even in adults when clinicians require repeated tests to monitor therapy.

Though we did not run any specific tests to evaluate ease of collection, we did not encounter any problem regarding sampling, storage or processing of DBS samples. None of the filter paper card showed any signs of absorption of moisture during 6 months of storage. Also, DBS offered more convenience for sample storage in terms of space required and safety in handling as no risk of leakage or spillage was involved while handling these samples.

In terms of limitations, the study had a small sample size, and evaluation was done in a laboratory setting instead of a field study as the current effort was a pilot study. Despite the limitations, the study does provide baseline data on the usefulness of the DBS technique, and recommends filed studies with larger sample size to further validate the findings of the current study.

Conclusion

DBS sampling correlated well with the conventional blood sampling method for serological and molecular testing. However, prolonged storage reduced the sensitivity of molecular tests, particularly for HCV RNA.

Disclaimer: The text was presented at the annual conference of the Pakistan Society for Study of Liver Diseases, and at the 1st International Conference of the Abbottabad University of Science and Technology. It received the Best Paper award at both forums.

Conflict of Interest: None.

Source of Funding: Pakistan Health Research Council.

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