International Journal of Biomedical and Advance Research
ISSN: 2229-3809 (Online); 2455-0558 (Print)
Journal DOI: https://doi.org/10.7439/ijbar
CODEN: IJBABN

Original Research Article

Dried tube specimens: A tool to ensure effective proficiency testing & quality control of Hepatitis B virus diagnosis in developing countries

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***Article History:**
Received: 01/06/2017
Revised: 10/06/2017
Accepted: 10/06/2017
DOI: https://doi.org/10.7439/ijbar.v8i6.4194

Abstract

Background: Proficiency testing (PT) is a key component of Quality assurance to evaluate the laboratory performance. Although HBV testing has rapidly expanded worldwide, but in resource limited settings (RLS) like India there is still a lacunae in PT programs to monitor and improve the quality of testing. Conventional PT programs use serum or plasma specimens that require strict settings for storage and transportation. Thus the aim was to standardize dried tube specimens (DTS) technique for stability studies of HBV specimens as has been done for PT programs in HIV-EQAS.
Methods: Dried tube specimens, an unusual, simple and easy to use technique, were standardized that can help assessing the quality of Hepatitis B Surface Antigen (HBsAg) testing in RLS. DTS were prepared by drying 30µl of samples mixed with 0.1% of green dye, left overnight at room temperature. The DTS were rehydrated with 250µl of PBS–Tween buffer before testing. Twenty DTS samples (09 HBV positive and 11 negative) were used for performing ELISA and RAPID tests at weekly interval for Dried tube specimens stored at 4ºC, 25ºC, 37ºC & 45ºC.
Results: Stability studies showed that HBsAg in the DTS specimens were stable upto 28 days at temperature 4°C, 25°C, 37°C & 45°C. We observed consistent and expected results for all the DTS samples by ELISA and RAPID tests for the different durations and temperatures. No difference in the qualitative nature of the HBV reactive and non-reactive samples were observed for Dried tube specimens over 28 days duration and stored at 4ºC, 25ºC, 37°C & 45°C temperatures. However, a decline in HBsAg levels were observed after four weeks (28days) for a few specimens stored at 45ºC.
Conclusion: Stability results of this preliminary study indicated that the reactivity status was unchanged and maintained for HBV plasma samples upto 28 days at temperatures (4ºC, 25ºC 37°C & 45°C) in the dried tube specimens. Further, we found DTS is an easy and simple method to prepare and transport quality control specimens for effective implementation of PT programs to monitor HBV testing in India.
Keywords: Proficiency testing (PT), Dried tube specimen (DTS), Hepatitis B Virus (HBV).

1. Introduction

Hepatitis B is a very common infection originally known as "Serum Hepatitis"[1]. Infections by the Hepatitis B virus present serious health problems in all parts of the world. The replication of Hepatitis B virus takes place in the liver after which the virus spreads to the blood where virus-specific proteins/antigens and their corresponding antibodies are found circulating in the plasma of infected people. The hepatitis B surface antigen (HBsAg) is most frequently used to screen for the presence of this infection. It is the first detectable viral antigen to appear during

infection. Different immunodiagnostic screening tests such as RAPID test and Enzyme immunoassay (EIA) use plasma or serum to detect the presence of these proteins/antigens and antibodies as means to diagnose the infection. Early detection and confirmation of the infection is essential to reduce the spread of the disease for which the blood samples sometimes may require transportation in cold chain, this is difficult in developing countries, where the modern facilities for the collection, storage and transportation of the samples at appropriate temperatures are either not developed or broadly not in practice. Efficiency of assay operator has a significant impact on the reliability of diagnostics test, which can be optimized by implementing a standardized proficiency testing programs like EQAS [2]. The regulation of the quality of diagnostic tests can have a significant impact on the quality of testing being provided to patients, and the lack of regulation can compromise clinical care [3]. Inadequate quality assurance and minimal or inappropriate training present limitations to the usefulness and cost effectiveness of non-laboratory based rapid diagnostic testing programs [4-6]. Thus implementation of quality management can effectively increase the confidence in the testing process by monitoring outcomes through quality assurance programs to minimize the variability and increase the accuracy of interpretation [4,5,7-9]. Laboratory quality assurance is a set of activities for ensuring quality in the processes by which products are developed and monitors preanalytic, analytic, and post analytic aspects of the testing process using two main methods [2]. Quality control (QC) ensures that an analytical phase i.e. actual laboratory testing is correct and error free [8,10,11]. External quality assessment (EQA), also called proficiency testing, is the tool used to assess the testing process independently. Importantly, EQA can not only be used to monitor technical performances but also identifies training opportunities based on proficiency results [2,4,9]

To overcome the logistics difficulties involved in transporting liquid plasma, simple, cost-effective and user friendly approach, based on usage of dried plasma was first developed for preparation of HIV Proficiency testing (PT) panels [12] it was observed that, the method of preserving the total solids of plasma or serum by removal of the water component by drying represents the most stable form of the storage of the important blood components. Dried Tube Specimen (DTS) does not require freezing of the samples at low temperatures such as –20^{o}C for storage and transportation; it is a cold chain independent approach and diminishes the transportation cost. Thus, DTS can be effectively used in External quality assurance Scheme (EQAS) in Proficiency testing (PT) [12].

The numerous advantages of DTS over liquid specimen led to plan the study on stabilization of DTS for Hepatitis B testing panels in resource limited settings like India to evaluate the stability of HBsAg in serum specimens stored at different temperatures for various lengths of time.

Therefore, this preliminary study was undertaken to analyse the stability of Hepatitis B virus in dried tube specimen. The study was performed to analyse stability of HBsAg in the DTS over 5 different days i.e. on 0th day, 7th day, 14th day, 28th day, and 42nd day at four different temperatures which include 4ºC, 25ºC, 37ºC & 45ºC.

2. Materials & Methods

2.1 Optimization of the sample volume to prepare DTS for HBV infected serum samples

The optimum volume required for preparing the DTS for HBsAg detection was firstly determined using rapid assays. Standardization of the optimal sample volume was performed using a well characterized panel of 4 samples out of which 2 were reactive and 2 were nonreactive. Sample volume of 20µl, 30 and 40µl were aliquotted in micro centrifuge tubes and were left open for air dry at room temperature in the bio safety cabinet for duration of 24 hour. On the next day, all the dried sample were rehydrated with 0.25 ml PBS Tween Buffer (0.1%, pH 7.4) (M/s SIGMA, USA), and were mixed gently and left overnight to allow complete solubilisation of the dried specimen. The tubes containing the rehydrated sera were gently mixed on the next day and tests were performed as per the manufacturer's instructions using three different RAPID test kits namely Vikia HBsAg rapid card test (M/s. Biomeuriex, France) and Hepacard One step visual RAPID test kit (M/s. Diagnostic Enterprises, Parwanoo) & Artron one step HBsAg RAPID kit (M/s. Artron Bioresearch, Burnaby Canada).

2.2 Preparation of DTS (Dried tube specimen):

A "20 -member panel" (9 HBV positive and 11 HBV negative) was used to prepare several sets of DTS specimens. After optimizing the volume of sera, DTS specimens were prepared by transferring 30µl of serum or plasma, premixed with 0.1 % (v/v) green dye (food color), into a 2 ml centrifuge tube. Green dye allowed visualization of the colored pellet at the bottom of the tube and did not interfere with the test results. The tubes were left open in a bio safety cabinet overnight to dry [12]. Twenty aliquots were made for each sample and were labelled appropriately. All the samples were then kept on the respective temperature as written on the label and arranged on the basis of day on which it was to be tested.

A day before testing, dried tube specimens were rehydrated using 250µl PBS–Tween resulting in approx 1:8 dilution of the original specimen but was considered as undiluted for the purpose of further testing. Specimens were gently mixed without vortexing for complete solubilization

of dried serum or plasma into the PBS buffer and then allowed to dry overnight at room temperature.

Multiple sets of dried tube specimens were prepared using a "20 -member panel" (9 HBV positive and 11 HBV negative) and stored at 4 different temperatures 4ºC, 25ºC, 37ºC & 45ºC. To evaluate the stability of HBsAg in the dried samples, one set from each temperature was rehydrated a day before testing on 0th day, 7th day, 14th day, 28th day and 42nd day and tested for the presence of the HBsAg using Rapid immunoassays (viz. Vikia HBsAg rapid card test (M/s. Biomeuriex, France) and HepaCard HBsAg rapid card test (M/s. Diagnostic enterprises, India) and ELISA Imunoassays (viz. Hepanostika HBsAg Ultra, M/s. Biomeriuex, France). The samples used in the study were ethically approved from the institutional ethics committee.

3. Results and Discussion

Nearly one third of the world's population has been infected with Hepatitis B and the virus is endemic in Asian countries. Thus HBV testing is the critical step for prevention of infection along with counselling of Hepatitis B virus infected people. Proficiency testing is lacking in many resource-limited settings because of the logistic difficulty of panel preparation & implementation and storage which requires very large infrastructure and facilities which are not always available. This lead us to plan our study on a newer approach employing dried plasma [12] instead of liquid plasma and testing the stability of Hepatitis B surface antigen (HBsAg) present in dried plasma obtained from the Hepatitis B infected patients.

Out of all the volumes (20µl, 30µl, 40µl) used for preparation of DTS, 30µl sample volume was found to be optimum for DTS preparation since it dried overnight and the results obtained during rapid testing in the form of bands were dark and more clearly visible. All subsequent studies were conducted using the DTS prepared with 30µl volume of plasma. Volume of 40µl was not selected as it took 12 hours more to get dry and also the bands obtained in the 40µl were comparable with that of 30µl in clarity. Further the bands were sharper with 30µl volume than 20µl. Thus we observed that 30µl of sera used for DTS preparation may facilitate proficiency testing study of HBsAg for EQAS in resource limited settings.

The result of three RAPID test kits using 20µl, 30µl & 40µl of sample volume for optimization in terms of Reactive (R) and Non-reactive (NR) have been shown in Table 1 and found to be expected.

Further the results of RAPID test & ELISA conducted to check the stability of 20 DTS specimen panel members which were stored at 4 different temperature (4ºC, 25ºC, 37ºC, 45ºC) and tested at 5 different time points (Day 0, Day 7, Day 14, Day 28 & Day 42) using the kits Vikia HBsAg rapid card test, (M/s Biomeuriex France) and Hepa card HBsAg rapid card test (M/s Diagnostic enterprises, Parwanoo, India) and the ELISA results using the kit Hepanostika HBsAg Ultra, (M/s. Biomeriuex, France) have been summarized in Table 2a & 2b. Cut off values were determined as per manufacturer's instruction and S/CO (E-ratio) were calculated for ELISA test.

When tested by RAPID test all negative specimens showed clear negative results for all sample duration and temperatures.

The results obtained from the preliminary study conducted indicates that there is no change in reactivity status of the sample when tested by ELISA based kits at low or high temperature (i.e. 4°C, 25ºC, 37ºC, 45ºC) upto four weeks. However, at higher temperature at or above 37ºC for sample Sr. No 5, 7 & 9 there was decline in the absorbance value beyond four weeks but still did not affect the reactivity status of the sample. When tested by RAPID test kits the results were very clear at temperatures 4ºC, 25ºC 37ºC even on the 42nd day but at high temperature above 37ºC test the above mentioned reactive samples came out to be non-reactive on 42nd day at higher temperature of 45ºC. This suggests that HBsAg levels were stable at 4ºC, 25ºC and 37ºC till 42nd day and the observed variations in HBsAg levels were within the variability of the RAPID assay and did not affect the expected results, except at 45ºC.

The decline in HBsAg levels observed after four weeks for above said three specimens were consistent in ELISA and rapid testing giving no test bands for otherwise reactive samples at 45ºC. This decline in HBsAg levels at higher temperature of 45ºC above four week duration can be corroborated on larger sample size.

Our preliminary data indicates that there could potentially be some loss of HBsAg in DTS after four weeks when DTS were stored at temperatures above 37ºC. But the results obtained from the experimental data on the 42nd day conclude that 3 of the reactive samples which gave light bands at 37ºC gave nonreactive result on 45 ºC. Therefore it is concluded that DTS containing HBV infected dried plasma is approximately stable for four weeks at all temperatures ranging from 4ºC-45ºC and stable for 5-6 weeks when kept at 4ºC-37ºC.

Thus DTS is a reliable alternative to serum collection and provides an effective approach for implementing Proficiency Testing programs in most serology testing for the detection of HBsAg as already been done in case of HIV serology [12]. This approach uses 8-10-fold less volume of specimen than other proficiency testing programs, which uses plasma volumes from 0.2 ml to 0.5 ml. DTS specimens, once rehydrated, can be tested by RAPID tests or EIA, and thus can be used for performing

HBV testing at different levels i.e. testing sites and laboratories. Further the method described in the article has the potential to be used for other types of rapid assays after due standardization.

As discussed in the preceding sections DTS has several advantages and can thus be used for the transportation of the samples during EQAS/PT and other testing programs. It is safer and less bio hazardous than liquid specimens. In addition, the specimens are stable at temperatures ranging from 4ºC to 37ºC as expected in many countries, especially during transport, and hence can be transported at room temperature without the need for maintaining an expensive cold chain. Further, the specimen remains stable at room temperature for few days once it is received at testing center.

Table 1: Shows the result of three RAPID test kits using 20µl, 30µl & 40µl of sample volume for optimization (where R indicates the reactive sample and NR indicates the non-reactive sample and + indicates the presence of control band)

S. No	Sample ID	Kit	Expected result	Result 20µl		Result 30µl		Result 40µl	
				Control	Test	Control	Test	Control	Test
1	Sample A	Kit 1	R	+	R	+	R	+	R
		Kit 2	R	+	R	+	R	+	R
		Kit 3	R	+	R	+	R	+	R
2	Sample B	Kit 1	R	+	R	+	R	+	R
		Kit 2	R	+	R	+	R	+	R
		Kit 3	R	+	R	+	R	+	R
3	Sample C	Kit 1	NR	+	NR	+	NR	+	NR
		Kit 2	NR	+	NR	+	NR	+	NR
		Kit 3	NR	+	NR	+	NR	+	NR
4	Sample D	Kit 1	NR	+	NR	+	NR	+	NR
		Kit 2	NR	+	NR	+	NR	+	NR
		Kit 3	NR	+	NR	+	NR	+	NR

Table 2a: Summarizes the reactivity status of DTS specimens stored at various temperature viz. 4°C, 25ºC, 37ºC, 45ºC and time duration i.e. 0th day, 7th day, 14th day, 28th day and 42nd day using the RAPID kit Vikia Hepanostika HBsAg Ultra, (M/s Biomeriuex France)

Sample Id	Expected Result	Day 0				Day 7				Day 14				Day 28				Day 42			
		Temp 4°C	Temp 25°C	Temp 37°C	Temp 45°C	Temp 4°C	Temp 25°C	Temp 37°C	Temp 45°C	Temp4°C	Temp 25°°C	Temp 37°C	Temp 45°C	Temp4°C	Temp 25°C	Temp 37°C	Temp 45°C	Temp4°C	Temp 25°C	Temp 37°C	Temp 45°C
1	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
2	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
3	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
5	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	NR
6	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
7	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	NR
8	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
9	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	NR
10	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
11	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
12	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
13	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
14	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
15	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
16	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
17	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
18	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
19	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
20	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

Table 2b: Summarizes the S/CO values of ELISA done using the kit Hepanostika HBsAg Ultra, (M/s Biomeriuex France) for the DTS kept stored at various temperature viz. 4°C, 25ºC, 37ºC, 45ºC and time duration i.e. 0th day, 7th day, 14th day, 28th day and 42nd day (S/Co value ≥1 indicates reactive sample (R) and S/Co<1 indicates non-reactive sample (NR))

Sample Id	Expected Result	Day 0				Day 7				Day 14				Day 28				Day 42			
		Temp 4°C	Temp 25°C	Temp 37°C	Temp 45°C	Temp 4°C	Temp 25°C	Temp 37°C	Temp 45°C	Temp4°C	Temp 25°C	Temp 37°C	Temp 45°C	Temp4°C	Temp 25°C	Temp 37°C	Temp 45°C	Temp4°C	Temp 25°C	Temp 37°C	Temp 45°C
1	R	22.5	21.1	24.5	22.6	56.0	55.7	71.7	66.0	49.2	52.2	50.3	50.1	40.2	46.6	45.6	43.5	45.4	45.5	40.0	44.4
2	NR	0.2	0.1	0.1	0.0	0.4	0.2	0.5	0.6	0.1	0.1	0.6	0.1	0.3	0.8	1.0	0.7	0.6	0.5	0.5	0.5
3	R	21.6	22.3	24.3	24.4	53.0	55.8	75.1	61.7	51.7	57.4	49.0	47.1	40.9	41.1	40.5	44.5	45.0	41.8	42.9	41.5
4	R	20.6	23.2	22.5	24.6	62.8	65.7	67.5	66.7	55.7	58.4	58.1	50.0	45.7	43.8	46.4	42.9	46.6	45.9	44.4	45.2
5	R	20.3	22.7	22.0	24.1	56.0	60.0	60.8	61.5	55.0	47.6	56.0	48.0	42.9	44.2	43.6	42.1	44.4	41.9	18.5	2.4
6	R	21.3	23.8	25.1	23.2	58.3	49.4	63.4	67.2	48.0	48.0	49.9	50.7	39.7	43.6	40.3	41.2	45.0	43.4	41.1	43.3
7	R	16.8	28.2	23.2	19.3	62.1	59.5	60.8	65.3	46.9	52.4	60.3	50.0	40.9	41.7	40.4	46.5	44.0	43.4	14.7	1.6
8	R	19.7	26.5	23.8	19.7	62.7	63.3	64.3	67.3	53.3	52.7	50.5	47.6	40.2	44.8	41.6	47.6	47.1	46.0	47.9	49.2
9	R	17.7	20.4	24.0	22.3	61.2	56.0	68.0	70.7	51.3	51.4	53.2	53.1	42.1	44.5	41.3	42.1	42.4	43.0	13.1	1.6
10	NR	0.1	0.3	0.1	0.0	0.3	0.3	0.3	0.6	0.1	0.5	0.6	0.4	0.8	0.5	0.6	0.5	0.5	0.4	0.5	0.5
11	NR	0.1	0.2	0.1	0.0	0.5	0.4	0.7	0.6	0.1	0.1	0.1	0.6	0.8	0.4	0.7	0.7	0.5	0.5	0.5	0.6
12	NR	0.1	0.2	0.1	0.0	0.6	0.5	0.5	0.3	0.1	0.1	0.1	0.6	0.4	0.4	0.6	0.5	0.5	0.5	0.8	0.6
13	NR	0.0	0.3	0.1	0.0	0.6	0.5	0.6	0.6	0.1	0.6	0.1	0.7	0.7	0.7	0.5	0.7	0.8	0.8	0.5	0.6
14	NR	0.1	0.3	0.0	0.0	0.6	0.5	0.5	0.2	0.7	0.6	0.1	0.7	0.4	0.6	0.5	0.7	0.5	0.5	0.6	0.7
15	NR	0.2	0.0	0.1	0.0	0.4	0.5	0.8	0.3	0.7	0.5	0.5	0.5	0.4	0.6	0.6	0.6	0.6	0.4	0.4	0.7
16	NR	0.0	0.4	0.1	0.0	0.6	0.4	0.6	0.5	1.0	0.1	1.0	0.1	0.5	0.8	0.7	0.8	0.9	0.6	0.6	1.0
17	NR	0.1	0.0	0.1	0.0	0.4	0.6	0.6	0.5	0.7	0.6	0.9	0.1	0.4	0.7	0.6	0.7	0.5	0.6	0.6	0.8
18	NR	0.0	0.4	0.1	0.0	0.6	0.4	0.3	0.4	0.8	0.1	0.9	0.1	0.3	0.6	0.3	0.7	0.6	0.5	0.5	0.6
19	NR	0.2	0.0	0.0	0.1	0.3	0.5	0.6	0.5	0.7	0.6	0.9	0.4	0.6	0.5	0.6	0.6	0.4	0.4	0.4	0.5
20	R	19.9	27.3	23.9	22.4	59.1	54.9	64.1	62.6	47.5	51.6	51.5	50.1	42.5	42.9	42.8	42.1	45.3	40.1	47.4	44.5

4. Conclusion

DTS based PT program can be a crucial component of external quality assurance strategies for preparation of PT panels and QC materials for HIV, HCV and HBV diagnosis, in developing countries to monitor and improve the quality and accuracy of testing. Thus, DTS approach has the potential to implement PT programs to all testing sites in developing countries where the temperature varies from very low to 37 ºC and storage resources are limited.

Acknowledgement

Authors acknowledge the help of Immunodiagnostic Kit Laboratory staff for their assistance. Authors acknowledge the help of various blood banks of Delhi-NCR for providing the samples for study.

Conflict of interest

The authors have no conflict of interest.

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