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## Original Article

# Assessment of the suitability of the dried blood spot (DBS) samples for detection of Treponemal antibodies using *in vitro* assays

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***Background & objectives*: This study was performed to assess the suitability of dried blood spot (DBS) samples for the serological screening of syphilis.**

***Methods*: Two hundred paired DBS and plasma samples collected from six sexually transmitted infection (STI) clinics during the year 2023 were tested using three kits -*Treponema pallidum* haemagglutination assay (TPHA), Syphilis Total Ab and ErbaLisa Syphilis after standardization of the dilutions of the DBS elutes and considering the results of the paired plasma samples as a true status.**

***Results*: The TPHA showed 89 per cent sensitivity and 100 per cent specificity, the EIA-Syphilis Total Ab. Kit showed 100 per cent sensitivity and 97 per cent specificity, whereas ErbaLisa syphilis showed 89 per cent sensitivity and 100 per cent specificity. However, one kit Syphilis ELISA (Oscar Medicare Pvt. Ltd., New Delhi) did not show agreement with the paired plasma samples at any dilution and was not considered suitable for the testing of DBS samples. The agreement between the plasma and DBS results was found to range from 94.5 to 98.5 per cent with a kappa agreement score of 0.89 for TPHA, ErbaLisa, and 0.97 for ELISA-Syphilis Total Ab.**

***Interpretation & conclusions*: The findings of this study confirmed that the DBS samples can be used for the detection of anti-treponemal antibodies using the above-validated kits and thus may be a valuable tool in surveillance and epidemiological surveys conducted in India. The study also highlighted the need for validation of any plasma/serum syphilis antibody detection assay on DBS samples before using it on DBS samples**

**Key words** Dried blood spot - syphilis - validation

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Sexually transmitted infections (STIs) impose major health and economic burdens globally. Understanding the patterns of STIs in different geographic pockets is important for the efficient planning and implementation of STI control programmes[1]. It is known that bacterial STIs, and HIV acquisition and transmission can act synergistically, enhancing the acquisition and/or transmission of the virus among high-risk groups (HRGs), such as men having sex with men (MSM), transgender/*hijra* (TG/H), people who inject drugs (PWID) and female sex workers (FSW)[1], thus emphasizing the need for STI diagnosis and control in these groups.

According to the World Health Organization (WHO), syphilis [Causative agent-*Treponema pallidum* (TP)] is a curable and common STI across the globe, infecting around 10-12 million people each year[2,3]. Most infected individuals have no symptoms, and therefore, a serological test is required to diagnose the infection. The WHO estimated that 7.1 million new cases of syphilis are acquired worldwide every day[3]. The stigma associated with STIs, low clinic attendance, a lack of testing resources at the peripheral level of STI clinics, the absence of a shared STI registry, and the focus on syndromic management all contribute to an underestimation of the true prevalence of syphilis in India[4]. The National AIDS and STD Control Programme (NACP) of India reported syphilis prevalence ranging from 0.01 to 0.77 per cent in antenatal clinic attendees from different States, and 0.2 to 0.4 per cent among FSW and MSM[5,6]. All pregnant women must undergo screening for syphilis and HIV as part of the National Health Mission (NHM) of India at all primary health centres (PHCs), community health centres (CHCs) and other secondary/tertiary healthcare facilities[7]. The DCGI (Drug Controller General of India) and CDSCO (Central Drugs Standard Control Organization) in India also emphasise testing for syphilis among blood donors as a rise in syphilis cases particularly among the blood donors have been reported in India previously[8].

To eliminate/reduce the syphilis infections in the country, it is of utmost importance to screen individuals with high-risk behaviour and pregnant women for syphilis and blood donors so that they are linked timely to appropriate treatment and care. Hence, to integrate syphilis testing effectively into the programme, the efforts in decentralization of the testing and in making the simple, affordable, sensitive, and targeted testing methods available at peripheral clinics are important.

The various serological tests (treponemal and non-treponemal tests) available for detection of syphilis typically use either serum or plasma samples for testing[9]. These tests require the collection of blood samples by venipuncture, a centrifuge for serum/plasma separation, a shaker, and refrigeration for the storage of reagents. As a result, these tests are inappropriate for use in rural or remote locations where the availability of adequate infrastructure may be a challenge.

The use of dried blood spot (DBS) samples for testing is a promising alternative to serum/plasma samples due to their ease of collection, storage, and transportation. Serological surveillance or diagnosis for various pathogens using DBS has been reported in earlier studies. In surveys, the collection of DBS has grown in popularity as a less expensive option compared to venipuncture[10, 11].

In India, the DBS samples are being used as a convenient sample collecting method for various surveys such as HIV Sentinel Surveillance (HSS) in HRGs, National Family Health Surveys, and Integrated Biobehavioural Study (IBBS). DBS samples have been used earlier in a few studies for syphilis serology[12-14]. Studies on DBS samples using syphilis serology demonstrated sensitivity and specificity ranging from 50 to 100 per cent[15]. However, since the manufacturer methodologies are not available for the use of the DBS samples, the findings cannot be generalized, and the local validation and standardization of procedures are necessary.

Since DBS has not been utilized or evaluated for syphilis testing in India, the present study was designed to validate the commercially available serological assays [TPHA (Treponema pallidum Haemagglutination assay) and EIA (Enzyme Immunoassay)] for the detection of anti-treponemal IgG antibodies in DBS samples.

## Materials & Methods

This study was conducted at the department of Microbiology, ICMR-National Institute of Translational Virology and AIDS Research, Bhosari, Pune between March 2023 to July 2023 after obtaining ethical clearance.

*Sample size*: Sample size was determined using the formula for hypothesis testing of a population proportion (here sensitivity/specificity) as mentioned in the WHO manual for sample size determination[16]. Considering a five per cent level of significance and 80 per cent power to detect a four per cent difference from the anticipated 100 per cent value of sensitivity and specificity, the sample size was determined as 92,

**Table I.** Different combinations of DBS elute with sample diluent used for TPHA and EIA

| S.No. | Name of kit | Dilution of plasma (as per the kit insert) | | Dilutions of the DBS | | Result of testing (% concordance) |
|---|---|---|---|---|---|---|
| | | Volume of sample diluent (µl) | Volume of plasma (µl) | Volume of sample diluent (µl) | Volume of elute (µl) | |
| 1 | TPHA | 190 | 10 | 170 | 30 | 80 |
| | | | | 150 | 50 | 80 |
| | | | | 125 | 75 | 100 |
| 2 | ELISA-Syphilis Total Ab. | 0 | 50 | 0 | 50 | 100 |
| 3 | ELISA-Erba Lisa | 25 | 125 | 120 | 30 | 80 |
| | | | | 100 | 50 | 80 |
| | | | | 75 | 75 | 80 |
| | | | | 25 | 125 | 100 |
| 4 | ELISA-Oscar | 50 | 100 | 120 | 30 | 70 |
| | | | | 100 | 50 | 70 |
| | | | | 75 | 75 | 70 |
| | | | | 50 | 100 | 70 |

TPHA, treponema pallidum haemagglutination assay; ELISA, enzyme linked immunosorbent assays; EIA, enzyme immunoassays; DBS, dried blood spot

rounded off to 100. Hundred TPHA positive and 100 TPHA negative paired plasma and DBS samples were used for validation.

*Sample collection*: Patients visiting six STI centres for care and treatment (Apex Regional STD Centre, VMMC, and Safdurjung Hospital, New Delhi, Maulana Azad Medical College, New Delhi, School of Tropical Medicine, Kolkata, Regional STI/RTI Training, Research and Reference Laboratory, TNMC & BYL Nair Ch. Hospital, Mumbai, Designated STI/RTI clinic, Civil Hospital, Satara and ICMR-NITVAR, Pune) were enrolled in the study after obtaining informed consent. Blood samples (10 ml) *via* venipuncture were collected in EDTA tubes from all the patients willingly participated in this study.

DBSs were prepared from the whole blood samples by adding 75 µl of whole blood on each of the five circles of the Whatman No. 1 filter paper card and dried at room temperature overnight. Completely dried DBS cards were stored at -20°C until their transportation to ICMR-NITVAR, Pune for subsequent testing.

At the collection and testing sites, the plasma was tested by the reverse algorithm (TPHA followed by RPR) as per the current guideline[17], and the results were reported to the patients for further treatment and care. The remaining samples were stored at -20°C until their transportation to ICMR-NITVAR.

*Kits for validation*: The following treponemal tests for syphilis were used for validation: TPHA (Bio-Rad Laboratories, Gemenos, France), EIA -Syphilis Total Ab (Bio-Rad Laboratories, Gemenos, France); EIA-ErbaLisa Syphilis (Transasia Biomedicals Ltd, Mumbai, India), and Syphilis ELISA (Oscar Medicare Pvt. Ltd. New Delhi, India).

*Study protocol*: The DBS cards were brought to room temperature before being used. A 6 mm disc was punched in the centre of the circle for each sample, saturated with the whole blood, and placed in an uncoated well of 96 well flat bottom microtiter plate. Sterile phosphate-buffered saline (PBS), 200 µl, was then added to each well, and the microtiter plate was incubated overnight at 2 to 8°C.

For the determination of the required DBS dilution after elution for each test, five TPHA-positive and five TPHA-negative paired plasma and DBS samples were used. Different dilutions of the DBS elute were prepared using the sample diluent from the respective kits and tested using the TPHA and EIA kits. The plasma samples were tested according to the manufacturer's instructions provided in the

kit inserts. The results obtained on plasma samples were considered as the true status of the sample. Table I shows various dilutions used and their results in comparison with the results obtained using the respective plasma samples.

The dilution showing 100 per cent concordance during initial standardization was used further for validation, which included 200 paired plasma and DBS samples (100 TPHA positive and TPHA negative). Positive and negative controls were included in each assay. A subset of samples (n=50, 25 TPHA positive and 25 TPHA negative) were tested for reproducibility/ repeatability with DBS samples.

*Inter-lab comparison (ILC)*: Twenty TPHA positive and negative samples were tested by all three validated kits at two different laboratories using the same procedure on plasma and DBS samples.

*Statistical analysis*: Percent agreement was tested by Cohen's kappa test statistic through 8 pairwise comparisons. The analysis was done using Stata 16.1 statistical software (Stata 16.1, StataCorp LLC, College Station, TX, USA)[18,19]. The qualitative results (positive/negative) obtained on paired DBS and plasma samples were compared. The discordance, sensitivity, and specificity of these kits using the DBS and plasma samples were calculated. The OD values distributions were tested for normality assumption by the Shapiro-Wilk test. In the case of non-normal data, the median comparison was done by the Mann-Whitney U test. The OD values were compared for plasma positive and DBS negative *vs*. plasma negative and DBS negative results. Similarly, plasma positive & DBS negative samples' OD values were compared with those of plasma positive and DBS positive samples. The median E ratios were compared by the Wilcoxon signed rank test.

## Results

*Standardization of the dilution of DBS elute for use in TPHA and EIA to detect treponemal antibodies*: A 100 per cent concordance between the antibody results from DBS and paired plasma samples was observed at the dilutions of DBS (125 µl sample diluent) + 75 µl elute for TPHA kit and at 25 µl sample diluent +125 µl elute (for ErbaLisa syphilis kit. A 100 per cent concordance was obtained for the EIA-Syphilis Total Ab. Kit when the DBS elute was used without dilution (neat; Table I). Thus, these dilutions were further used in the validation exercise. For the Syphilis ELISA kit from Oscar Medicare, only 70 per cent concordance was observed between the plasma and DBS results at all the dilutions (Table I). Hence, it was concluded that while this kit is useful for testing the plasma/serum samples for treponemal antibodies, it is not suitable for testing DBS samples and was not validated further.

*Validation of the TPHA (BioRad), EIA-Syphilis total Ab., BioRad and ErbaLisa syphilis with standardized DBS dilution*: Two hundred (100 TPHA positive and 100 TPHA negative) paired DBS and plasma samples were used for this study. The anti-treponemal antibody results of plasma samples were considered the gold standard. The agreement of the test results using DBS and plasma samples was 94.5 per cent for both TPHA and EIA-ErbaLisa Syphilis and 98.5 per cent for EIA-Syphilis Total Ab. Out of 200 samples, eleven (5.5%) showed discordant results (plasma positive; DBS negative) by EIA-ErbaLisa Syphilis and TPHA assays, and three (1.5%) showed discordant results (Plasma negative; DBS positive) by EIA -Syphilis Total Ab. The TPHA showed 89 per cent sensitivity and 100 per cent specificity, EIA-Syphilis Total Ab., kit showed 100 per cent sensitivity and 97 per cent specificity and ErbaLisa syphilis showed 89 per cent sensitivity and 100 per cent specificity. The kappa values ranged between 0.89 (TPHA and ErbaLisa) and 0.97 per cent (Biorad) (Table II). The median E ratios of these 11 samples were statistically significant (*P* value=0.001) for ErbaLisa Plasma and DBS both.

Plasma OD values in known positive samples were higher using the Syphilis Total Ab than with DBS. The median OD value for positive plasma samples was 3.45, while the median value for DBS-positive samples was 2.33 ($P<0.01$). Plasma OD values in known positive samples were higher using the ErbaLisa assay than with DBS. The median OD value for plasma was 3.71, while the median OD value for DBS was 0.899 (Mann Whitney U test $P<0.01$).

As per the Central Drugs Standard Control Organisation (CDSCO), Ministry of Health and Family Welfare, New Delhi, India criteria for the acceptance of syphilis kit, the sensitivity should be ≥85 per cent and specificity should be ≥93 per cent[20]. Hence, by this criteria, all three kits are suitable for use on DBS samples for the detection of anti-treponemal antibodies. Furthermore, the Inter Laboratory Agreement (ILA)

**Table II.** Per cent agreement between plasma & DBS results using Biorad, Erbalisa and TPHA assays

| Sr. No. | Name of the kit | Per cent agreement (%) | Cohen's kappa value for rater agreement (*P* value) | Per cent agreement in TPHA negative samples (%) | Per cent agreement in TPHA positive samples (%) | Sensitivity (95% CI) | Specificity (95% CI) |
|---|---|---|---|---|---|---|---|
| 1. | EIA-Syphilis Total Ab. (Bio-Rad Laboratories, Gemenos, France) | 98.5 | 0.97 (<0.001) | 97 | 100 | 100 (96-100) | 97 (91-99) |
| 2. | EIA-ErbaLisa Syphilis (Transasia Biomedicals Ltd, Mumbai, India) | 94.5 | 0.89 (<0.001) | 100 | 89 | 89 (81-94) | 100 (96-100) |
| 3. | TPHA (Bio-Rad Laboratories, Gemenos, France) | 94.5 | 0.89 (<0.001) | 100 | 89 | 89 (81-94) | 100 (96-100) |

showed 100 per cent concordance between the results obtained from both the laboratories.

## Discussion

This study reports that all three commercially available kits *viz*, TPHA, ELISA-Syphilis Total Ab, ErbaLisa Syphilis, were found suitable for detection of treponemal antibodies in DBS samples.

The study showed acceptable 94.5 per cent and 100 per cent sensitivity and specificity for TPHA, respectively; 100 per cent and 98.5 per cent for ELISA-Syphilis Total Ab.; and 94.5 per cent and 100 per cent for ErbaLisa Syphilis. One of the earlier reports mentioned poor performance with TPHA (sensitivity of 50.5%) and EIA (both kits from Lab21 Syphilis Total Antibody EIA, Lab21 healthcare, Kentford, UK) (specificity 50.4%) but good 95.5 per cent sensitivity and 99 per cent specificity for the *Treponema pallidum* particle agglutination assay (TPPA) (Fujirebio, Tokyo, Japan) kit[21]. In another study, the anti-Treponemal antibody (*T.pallidum* Ig) test (*Treponema pallidum*, Biokit 3.0, Barcelona, Spain) performed on DBS showed 93 per cent sensitivity and of 99 per cent specificity[21-23]. The present study showed good TPHA and EIA performance using DBS samples when compared with paired plasma samples. An ideal diagnostic assay exhibits high sensitivity and specificity, thereby efficiently detecting majority of true cases while accurately excluding false negatives. However, the TPHA and ErbaLisa Syphilis showed a lower sensitivity as compared with the Syphilis Total Ab., TPHA and ErbaLisa Syphilis found 11 out of 100 plasma-positive specimens negative, which may be the result of a small concentration of anti-treponemal antibodies present in the DBS samples. Only three out of 100 plasma negative specimens were found to be positive by Syphilis Total Ab. We found that all the three respective patients were recently vaccinated with Hepatitis B vaccine and the false positive may have been due to this vaccination. This demonstrates that the testing of DBS samples may not pose a severe problem of false positive results.

This study indicates the usefulness of DBS as a feasible alternative to serum/plasma samples for serological detection of syphilis. The relative ease of sample collection, transport, and storage are significant benefits of DBS which makes it amenable for use in field settings, including epidemiological surveys.

There are few limitations to this study. Firstly, the samples were collected from STI centres, which are located in major towns and cities and transported in controlled conditions, which was not a major concern. Hence, the stability testing of DBS samples during transportation and storage conditions were not conducted; however, such issues are likely to be encountered in low resource settings. Secondly, as DBS samples are not currently validated for non-treponemal tests, it is not possible to differentiate between current and past infection, which has an important bearing on the clinical management.

Despite these limitations, DBS testing may help overcome barriers to testing in the communities not reached by traditional healthcare services and for epidemiological studies.

Overall, validation of all three laboratory assays (TPHA-BioRad, EIAs-BioRad and Transasia Biomedicals) for detection of anti-treponemal

antibodies using DBS showed satisfactory results as per the CDSCO criteria. Since the sensitivity of ELISA manufactured by Bio-Rad Laboratories was found to be better than the other two kits, it is suggested that this kit may be considered as the first choice for testing of DBS samples for detection of anti-treponemal antibodies for HSS, STIs, and other seroepidemiological surveys conducted in the country.

Considering that treponema-specific antibodies may be detectable in patients with either active syphilis or a past successfully treated infection. It would be imperative to review the patient's clinical and treatment history while interpreting the results of treponemal serology.

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***Conflicts of Interest*****:** None.

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