

Performance validation of western blot for anti-HIV antibody detection in blood samples collected on filter paper (DBS)

Validação de desempenho do western blot para detecção de anticorpos anti-HIV em amostras de sangue coletadas em papel-filtro (DBS)

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ABSTRACT

Introduction: For conducting field studies on human immunodeficiency virus (HIV) serodiagnosis, the use of samples collected on filter paper [dried blood spot (DBS)] is profitable owing to the simplicity in handling and delivering them. Because of these characteristics, the use of DBS is recommended for studies in which the goal is to increase the population access to diagnostic testing, including HIV infection screening. For HIV diagnosis, the conventional strategy firstly uses an enzyme immunoassay as screening test, and the positive sample is confirmed on a complementary assay, as western blotting (WB). **Objective:** This study aimed at evaluating the analytical performance of WB assay for analyzing DSB samples. **Method:** One hundred eighteen blood samples collected in filter paper were analyzed by a modified WB. These samples derived from the SampaCentro Study, and they were HIV-antibody-positive in a screening test. In order to assess the reliability of these results, the assay performance was previously certified by employing the sample panel (reference panel) of Instituto Adolfo Lutz (IAL), which consisted of whole blood and plasma paired samples. **Results:** All of the DBS samples [100% (118/118)] showed the band pattern compatible with the criterion for HIV positivity established by the Ministry of Health. To validate the WB testing, the reciprocal relationship between antibody reactivity of DBS samples and of the respective plasma samples was investigated. **Conclusion:** WB optimization allowed the achievement of accurate results, and the easy DBS sample collection, transportation and storage will support the use of this immunodiagnostic technique for conducting HIV/acquired immunodeficiency syndrome (Aids) epidemiological surveys.

Key words: HIV antibodies; western blotting; validation studies; dried blood spot testing; Aids serodiagnosis.

INTRODUCTION

It is essential for the world health community to find ways to simplify and improve the accuracy of human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (Aids) diagnosis, without reducing the quality of care provided to patients⁽¹⁾.

An expanded HIV diagnostic coverage is a challenge in Brazil, due to its vast territory and the substantial number of isolated populations⁽²⁾. Access to diagnosis is crucial, allowing referral of the individual to prevention and treatment services to avoid spread of the virus and to improve quality of life⁽³⁾.

Identification of HIV infection by laboratory tests is based mainly on detection of specific antibodies in biological samples collected from patients and/or individuals with behavior that put them at risk of acquiring this infection. Several types of samples may be used in the laboratory diagnosis of HIV infection, such as plasma, serum, whole blood, oral fluid, and blood samples collected on filter paper filter [dried blood spots (DBS)] and saturating it. Specimen choice depends on logistics, the selected population, the HIV testing strategy, and the algorithm of laboratory tests⁽⁴⁾.

Epidemiologic studies can be hampered by the difficulty in obtaining adequate blood samples, mainly from children and old people, and in storing aliquots of these materials in developing countries⁽⁵⁾.

DBS samples have become widely employed in the serological diagnosis of several illnesses because of facility in transportation, storage, and handling, as well as in seroepidemiologic studies of HIV in remote areas⁽⁶⁻⁸⁾. The use of these samples minimizes the risks associated to biological transportation and occupational accidents with potentially infected materials⁽²⁾.

In field researches, the employment of DBS samples is advantageous because they are easy to be handled and transported⁽⁸⁾. These characteristics place DBS technology at a prominent position in researches that aim at increasing people's access to diagnostic tests, including HIV tests, both serological and molecular.

Anti-HIV antibodies can be detected with precision in DBS samples⁽⁸⁾, which, when stored under controlled conditions of humidity and low temperature, are stable during at least 56 months⁽⁹⁾. Comparative analyses performed with serum samples and DBS in enzyme immunoassays (EIA) demonstrated strong correlation between the concentrations of antibodies in these samples, and these results suggest that the blood saturating filter paper may effectively substitute serum in anti-HIV assays^(10,11).

The conventional strategy for the diagnosis of HIV infection is using initially an immunoassay (screening test) and, when a positive result is produced, conducting a complementary assay, such as the western blot (WB), to confirm reactivity of the sample⁽¹²⁻¹⁴⁾. Although the quality of the available reagents has been increasingly enhanced, serum/plasma reactivity in screening tests still require confirmation by means of immune methods with more advanced technologies⁽¹⁰⁾.

In Brazil, procedures for the laboratory diagnosis of HIV infection are standardized by means of an ordinance by the Ministry of Health, which rules from the type of biologic material to be analyzed to the release of result reports⁽¹⁵⁾.

The development, the adaptation, or the implementation of an analytical method involves the evaluation process (usually named validation) to estimate its efficacy in laboratory routine⁽¹⁶⁾. The adaptation of the technique does not mean, necessarily, that it is correctly performed and it can provide valid results⁽¹⁷⁾. It is necessary to prove, by means of objective evidence, that the requirements for a certain application or specific usage were met⁽¹⁸⁾. Therefore, it is fundamental that laboratories have objective means and criteria to demonstrate that the used methods produce reliable results and ensure the quality, good reputation and credibility of their services⁽¹⁸⁾.

In the verification and validation of analytical methods adopted in laboratory routine, the minimum requirements, such as accuracy and precision, must be used to secure the achieved

performance. Similarly, in the use of a biological sample different from the recommended for the diagnostic reagent kit, previous validation is necessary to rule out the possibility of an effect induced by the sample in the result (matrix-induced effect). And any change in the existing technique procedure requires validation of its analytical performance⁽¹⁷⁾.

Likewise, it must be taken into consideration that reliability of laboratory diagnosis also depends on the pre-analytical phase, that is, collection, storage, time and transportation conditions of the sample to the laboratory^(17,19).

OBJECTIVE

This study was aimed at validating the analytical performance of WB used in the assessment of reactive DBS samples of SampaCentro Project.

METHOD

The study was approved by the scientific technical council of Instituto Adolfo Lutz (IAL) no. 0075-D-2010.

Considering the importance of confirming the reactive results detected in the screening assay for anti-HIV antibodies [ELISA/EIE Q-Preven HIV 1+2 – DBS (Symbiosis Diagnóstica Ltda., São Paulo, Brazil)] and due to the availability, at the HIV/Aids Laboratory – Immunology Center (CIM), IAL, São Paulo, Brazil (Lab HIV/Aids-CIM/IAL), of the WB reagent kit [Cambridge Biotech HIV-1 (Maxim Biomedical, Inc., Maryland, USA)], recommended for use in serum or plasma samples, a modified protocol was empirically applied to evaluate DBS samples.

Monitoring quality and analytical performance of the different WB lots was extremely important in the present study. In this context, the samples of reference panels SeraCare BBI Diagnostics [Boston Biomedica Inc. (BBI)], HIV-1 PRB205 (performance panel), and HIV-1 PRB972 (seroconversion panel) impregnated filter paper. The respective extracted eluates were employed to evaluate analytical sensitivity and specificity of WB performance.

DBS samples

One hundred eighteen blood samples collected on filter paper were analyzed, which were reactive in the assay ELISA/EIE Q-Preven HIV 1+2 – DBS, from the SampaCentro Study – Behaviors and sexual practices, access to prevention, HIV prevalence and other

sexually transmitted infections (STI) among gays, transvestites, and men who have sex with men (MSM) in the central region of the city of São Paulo, Brazil^(20, 21).

DBS samples were collected by fingerstick using a standardized collection kit (Bio-Oxford Importação Ltda., São Paulo, Brazil). Three to five drops of blood were deposited on filter paper Schleicher and Schuel - S&S 903 (Whatman/GE Healthcare Life Sciences do Brasil, São Paulo, Brazil) on a previously delimited area. The DBS sample of each individual was adequately identified with a unique code and, when completely dry, was stored at an individual envelope containing desiccant sachets (silica-gel). At Lab HIV/Aids-CIM-IAL, the samples were stored frozen at -70°C up to the performance of anti-HIV serological testing^(20, 21).

DBS sample collection, transportation and storage were carried out according to the instructions by the manufacturer of the diagnostic reagent kit Q-Preven HIV 1+2 – DBS (Symbiosis Diagnóstica Ltda., São Paulo, Brazil), based on orientations of the National Committee for Clinical Laboratory Standards (NCCLS)⁽²²⁾ and the World Health Organization (WHO)⁽⁴⁾.

Standardization of the elution technique

In order to analyze the 118 samples known to be to DBS HIV-reactive by WB, a circle (perforated edges) of 6 mm in diameter of each sample absorbed on the filter paper was cut out and eluted in blotting buffer (final dilution 1:200).

Twenty-five microliters of the kit control samples – nonreactive, weakly reactive, and strongly reactive serum for HIV – impregnated the filter paper Schleicher and Schuel - S&S 903 (Whatman/GE Healthcare Life Sciences do Brasil, São Paulo, Brazil). After the drying procedure, two circles were cut out of each control serum and eluted in 1ml of blotting buffer (final dilution 1:100). After elution, 1 ml of each sample and controls were transferred to the respective WB nitrocellulose strips. The other procedures of the reaction followed the manufacturer's instructions.

Validation of the modified WB procedure

Sample panel of whole blood and plasma of IAL – reference panel

At the conduction of SampaCentro Project, just the samples of dry blood (on filter paper) were analyzed, what made it impossible to carry out the comparative study between *in natura* serum/plasma samples (reference samples) and DBS samples. Thus, for the validation of the current study, it was necessary to use a

reference panel made of paired samples of whole blood and plasma with the following reactivity patterns: 20 HIV-negative samples and 20 HIV-positive samples (10 weakly reactive and 10 strongly reactive), followed by clinical and laboratory data of the respective individuals.

The whole-blood samples impregnated filter paper (DBS) and were, later on, evaluated (dilution 1:200) at WB, simultaneously with the plasma samples at the dilution 1:100, as recommended by the manufacturer of the diagnostic reagent kit.

Validation

In view of the modified test protocol, the validation process of the method was fundamental to ensure result reliability⁽¹⁶⁾. To that end, the following (minimum) requirements were adopted to validate WB performance: accuracy, precision (inter- and intra-assay), sensitivity of the diagnostic test and other considerations (limit of detection and matrix-induced effect)⁽¹⁷⁾.

Accuracy

Diagnostic accuracy was assessed in 20 DBS samples [seven HIV-positive (strongly reactive), six HIV-positive (weakly reactive) and seven HIV-negative]⁽¹⁷⁾, concomitantly with the respective plasma samples. The studied sample results were compared with the of the reference sample results⁽²³⁾.

Precision

With the availability of several samples of the IAL panel, we chose to assess intra- and inter-assay precision in a larger number of HIV-positive DBS than the procedure for qualitative assays described by Rabeau *et al.* (2007)⁽¹⁷⁾.

Intra-assay precision

Seven HIV-positive DBS samples (four strongly reactive and three weakly reactive) were analyzed in triplicates in the same reaction.

Inter-assay precision

Among the seven HIV-positive DBS samples used in the intra-assay evaluation, three (two strongly reactive and one weakly reactive) were selected to be analyzed in three distinct assays.

Sensitivity

The test sensitivity was determined by the analysis of 20 HIV-positive DBS samples (10 strongly reactive and 10 weakly

reactive), with confirmed clinical and laboratorial diagnoses of HIV infection.

Specificity

In order to verify specificity of the test, 20 HIV-negative DBS samples of the IAL panel were used.

Limit of detection

In order to estimate the assay limit of detection, a sample of HIV-positive whole blood was diluted in series (from 1:2 to 1:2048) in HIV-negative blood, and, afterwards, dilutions impregnated filter paper. Similarly, corresponding plasma sample dilutions were prepared. All the prepared samples were concomitantly analyzed in the same WB reaction run. The limit of detection was established as the highest sample dilution that has presented the specific bands to meet the positivity criterion for this virus⁽¹⁵⁾. The observed dilution was assessed in five replicates to confirm the detected band profile.

Matrix-induced effect

Because DBS has not yet been recommended as biological sample to be analyzed in the employed WB diagnostic reagent kit, the possibility of interference (matrix-induced effect) of the sample over the assay result was studied. To that end, nine DBS samples were analyzed, with the following reactivity results: six HIV-positive (three strongly reactive and three weakly reactive) and three HIV-negative⁽¹⁷⁾.

Result interpretation

According to the reactivity standards recommended by the producer of the respective reagent, qualitative interpretation of results was adopted, and they were expressed as positive, negative, or indeterminate for HIV.

Data analysis – validation process

Agreement of qualitative results obtained in DBS samples and in the respective plasma samples was evaluated by the statistic Kappa method⁽²⁴⁾.

The agreement levels, according to Kappa (k) values, were interpreted as proposed by Altman (1999)⁽²⁵⁾, adapted from Landis and Koch (1977). As reported by these studies, k value < 0.2 represents poor reproducibility, 0.21-0.4, fair; 0.41-0.6, moderate; 0.61-0.8, good; and 0.81-1, very good agreement.

RESULTS

Plasma samples forming commercial seroconversion PRB972 ($n = 6$) and performance PRB205 ($n = 25$) panels presented 100% (31/31) of agreeing results. In view of these results, the good assay performance can be determined in the analysis of eluates from panel samples impregnating filter paper.

Initially, the 118 HIV-reactive DBS samples of SampaCentro Project were diluted at 1:100, according to instructions by the WB standardized test manufacturer to analyze serum or plasma samples. However, when HIV-negative DBS samples were tested, some non-specific HIV bands of low intensity occurred. Based on this, new dilutions were prepared for HIV-reactive and HIV-negative DBS samples, and dilution 1:200 was considered ideal given the produced results of good sensitivity and specificity, respectively.

All HIV-reactive DBS samples (118/118) analyzed in the WB presented the same pattern of bands compatible with the positivity criterion for HIV established by the Ministry of Health, what demonstrates that the samples remained viable and adequate for the conduction of WB assays.

In the interpretation of results obtained in the assessment of diagnostic accuracy, the band pattern of DBS samples (HIV-positive and -negative), and that of plasma (reference sample) presented similarity (100%); there was no occurrence of false results.

In the assessment of WB diagnostic accuracy, the agreement degree between the results obtained in the 20 DBS samples (HIV-positive and -negative) and in the reference samples (plasma) was 100%, being the interpretation of $k = 1$ (very good). In **Figure 1**, results of accuracy are demonstrated in six studied DBS and plasma samples.

In the determination of precision, the maximum degree of agreement was verified ($k = 1$) of the results obtained from the analyzed samples in triplicate in the same reaction (intra-assay precision) and in three different assays (inter-assay precision). **Figure 2** shows, as an example, the result found in a HIV-positive DBS sample (weakly reagent) in the evaluation of intra- and inter-assay precision.

Regarding the characteristics of sensitivity and specificity of the test, the DBS samples analyzed in parallel with the corresponding plasma samples had their results reproducible ($k = 1$) both in HIV-negative samples (specificity) and in HIV-positive samples (sensitivity).

The limit of detection was defined as the dilution 1:64, in which the positivity criterion for anti-HIV antibodies was still

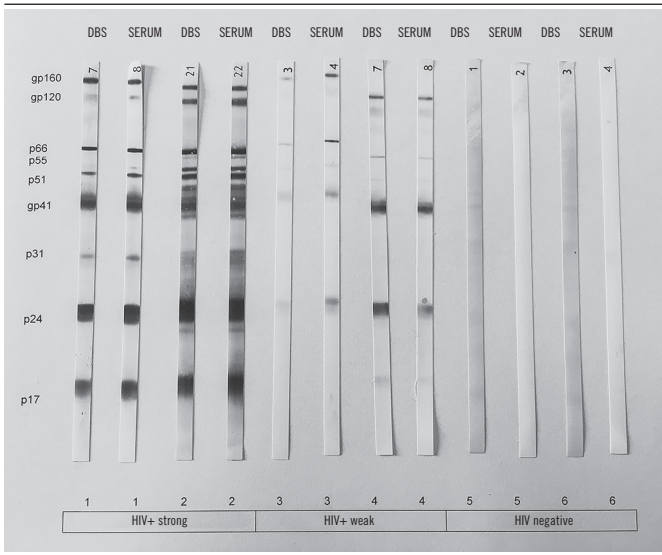


FIGURE 1 – Evaluation of accuracy in six paired DBS and plasma samples
 DBS: dried blood spot; +: positive.

present. Next, five replicates of the sample were analyzed, which confirmed the observed profile of bands – gp160 (low intensity), gp120 (low intensity), p66 (low intensity), p51 (low intensity), gp41, gp31 (low intensity) and p24, at the dilution 1:64. **Figure 3** presents the pattern of bands observed in the different dilutions (ratio 2) of a DBS sample and corresponding plasma in the reaction run to determine the limit of detection.

In the verification of the possible matrix-induced effect, the employment of DBS samples (eluate) at WB was demonstrated not to cause different results from those found in the respective plasma samples (reference sample) analyzed in parallel.

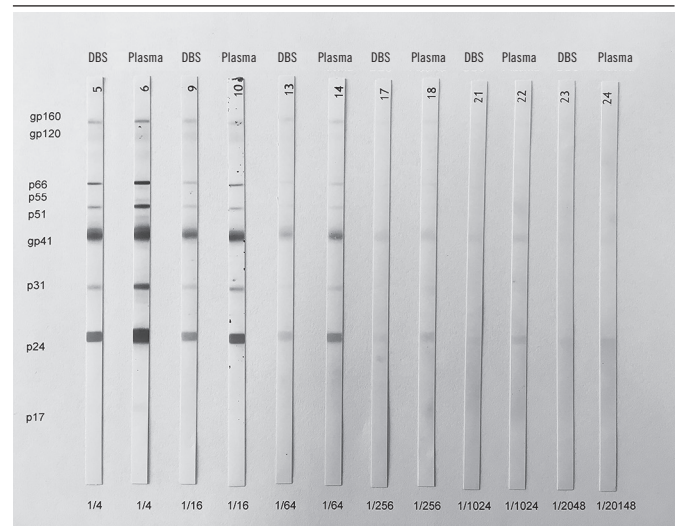


FIGURE 3 – Profile of bands detected in each dilution (ratio 2) of a DBS sample and corresponding plasma
 DBS: dried blood spot.

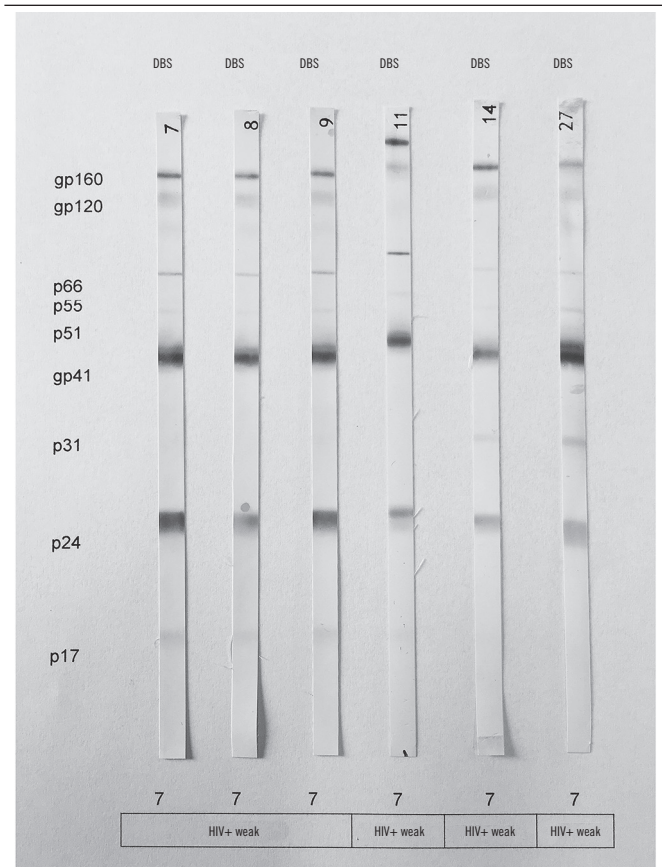


FIGURE 2 – Results of evaluation of intra- and inter-assay precision of an HIV-positive DBS sample (weakly reactive)
 DBS: dried blood spot; +: positive.

DISCUSSION

One must take into consideration that reliable diagnosis depends on pre-analytical aspects, as the choice of the correct sample and its conditions of transportation to the laboratory⁽¹⁷⁾. These and other procedures, such as drying and storage of DBS samples, followed the established protocol^(4, 21, 22) on the maintenance of material stability, and, consequently, the feasibility of HIV infection diagnosis. These variables are crucial sources of variation/error that become important during assay validation. The same practice was adopted with the panel prepared at CIM/IAL.

Validation is essential to define whether the developed methods are completely adequate to the objectives to which they are destined, in order to yield reliable results that can be satisfactorily interpreted⁽¹⁶⁾. The assessed parameters were judiciously learned

and adapted to the objectives of the validation study of the serological assay⁽¹⁷⁾. The HIV-positive DBS samples presented antibodies directed against the same viral proteins, and the intensity of the bands suggested the presence of a similar quantity of specific antibodies produced, when compared with the reactivity patterns exhibited by the equivalent plasma samples.

As cited by Varnier *et al.* (1988)⁽¹⁰⁾, in the present validation study, the anti-HIV serological result of DBS samples was comparable with that of plasma samples, what demonstrates that the whole blood impregnating filter paper was appropriate for diagnosis in the employed WB test. Thus, with the validation of WB analytical performance, DBS can be useful to confirm reactive results detected in the screening test, principally in those samples from great-scale HIV screening programs; there is still the advantage of using specific biological materials collected by fingerstick.

Blood samples of HIV-1-positive individuals, collected on filter paper (from November 2011 to January 2012)⁽²¹⁾ and stored until the conduction of the current study were not affected by environment conditions, neither were test results (positive or negative)^(8, 10) changed. These facts became evident when these samples were employed as part of a previous study⁽²¹⁾. All these DBS samples enclosed in individual envelopes containing desiccant sachets (silica-gel) and maintained frozen (-70°) kept their stability of anti-HIV-1 antibodies, even the samples with low positive reactivity (weakly reactive anti-HIV). Any alterations in their components would have been easily noted in the assay final result⁽²⁶⁾. No falsely negative or undetermined reactions occurred; neither did false positive results during the evaluation of HIV-negative samples from the IAL panel.

At the present study, the DBS samples (IAL panel) included in the validation test of the Western Blot Cambridge Biotech HIV-1 presented a heterogeneous reactivity profile, as previously described in the item Results. The analyte reactivity in DBS samples (positivity or negativity of anti-HIV antibodies) demonstrated that the use of WB for this aim was adequate. One can say that changing matrix caused no effect on the final result when in comparison with the reference sample.

This study presents important restrictions. One of them is associated with the employed biological samples, which came from SampaCentro Project. As this project planned collection and analysis of a single type of sample (DBS), in its conduction, it was impossible to assess and validate the comparative assays, using serum and plasma samples. The reason for the strategic blood collection on filter paper was that the individuals were recruited in public places, interaction spaces^(20, 21). Thus, we chose the least

invasive puncture technique in a way to conveniently obtain biological material for laboratory analyses. Blood collection was not carried out by venipuncture to obtain serum or plasma samples. Thus, in the development of the current work, we used the IAL panel. This material made of whole blood and plasma, characterized by reactivity of specific anti-HIV antibodies, was employed to validate the WB analytical performance. As a consequence, the results yielded in the DBS sample analysis of SampaCentro Project are reliable. Result comparability was established by evaluation of samples recommended by the manufacturer of the WB (plasma) and the DBS samples (modified WB assay), in parallel, to verify the eventual occurrence of alterations that could interfere with the study results. The modified WB assay was validated for the desired aim because sample results were similar and based on pre-established parameters.

Another important limitation was the remaining quantity of DBS samples to conduct this study. Therefore, there was no availability of this biological material to do the analysis of another confirmatory assay to compare results in different modalities of diagnostic techniques.

The achieved results reinforce the observations made by researchers⁽¹⁰⁾, what indicates that the blood collected on filter paper can effectively substitute serum or plasma samples in anti-HIV screening programs.

CONCLUSION

The set objectives, regarding the assessed performance parameters, met the suggested acceptance criteria.

Although further studies are necessary to evaluate a larger number of samples, in the present study the WB performance parameters were established, and the minimum acceptance requirements were fulfilled for the employment of DBS samples. WB was observed to yield reliable results as the assay was sensitive and specific. Moreover, the easiness to perform collection, transportation, and storage of DBS samples will be able to make the employment of WB technique viable in HIV/Aids epidemiological surveys.

ACKNOWLEDGEMENTS

The authors thank André R. Campos, of CIM/IAL, for the technical support in the conduction of this study. They also thank the members of the SampaCentro Study Group: Maria Amélia S.

M. Veras, Gabriela J. Calazans, Manoel Ribeiro, Márcia Giovanetti, Ricardo Gamboa, Isadora F. Lins, Regina Facchini, Carmem Aparecida F. Oliveira, Carlos Augusto V. Castro, Carmen Lúcia Soares, Edilene P. R. Silveira, Elaine L. Oliveira, Graça Ribeiro, Márcia Jorge Castejon, Rosemeire Yamashiro, Denise Andrade, Jucélia Barbosa, Margaret Dominguez, Mariângela Nepomuceno, Aline R. Barbosa, Cleiton Eduardo Fiório, Luiz Fabio A. de Deus,

Mariana L. Lisboa, Marina M. O. Pecoraro, Tiago R. Marin, Adriano V. Zago, Bianca Thais M. Pascoal, Brenner Y. K. C. Carneiro, Bruno Puccinelli, Camila V. B. Souza, Carolina Simone S. Adania, Cecília F. França, Félix Luis R. Silva, Higor M. Valente, Janaina Lima, Jeilson F. Lima, Luciana S. Almeida, Marilda Madalena Martins, Paulo Clécio S. Souza, Paulo Sérgio Stockler, Ana Paula Amaral and Deni Gomes.

RESUMO

Introdução: Em estudos de campo é vantajoso o emprego de amostras coletadas em papel de filtro (DBS) pela simplicidade em manuseá-las e transportá-las. Essas características colocam a tecnologia de DBS em posição de destaque nas pesquisas que buscam aumentar o acesso da população aos testes diagnósticos, incluindo os testes sorológicos para vírus da imunodeficiência humana (HIV). A estratégia convencional para o diagnóstico da infecção pelo HIV é utilizar um teste imunoenzimático como primeiro teste e, quando este for reagente, um teste complementar, como o western blot (WB), para confirmar a reatividade da amostra. **Objetivo:** Neste estudo foi avaliado o desempenho analítico do ensaio WB em amostras DBS. **Método:** As 118 amostras de sangue coletadas em papel-filtro, provenientes do estudo SampaCentro e reagentes para anticorpos anti-HIV no ensaio de triagem, foram analisadas no WB modificado. Para conferir a confiabilidade a esses resultados, o desempenho do ensaio foi previamente validado por meio de painel de amostras do Instituto Adolfo Lutz (IAL) (painel de referência), composto por amostras pareadas de sangue total e plasma. **Resultados:** Todas as amostras DBS [100% (118/118)] apresentaram o padrão de bandas compatível com o critério de positividade para HIV preconizado pelo Ministério da Saúde. Na validação do WB, foi averiguada a reciprocidade da reatividade dos anticorpos das amostras DBS e de suas respectivas amostras de plasma. **Conclusão:** A otimização do WB possibilitou a obtenção de resultados confiáveis, e a facilidade de execução, coleta, transporte e armazenamento de amostra DBS poderá viabilizar a utilização dessa técnica imunodiagnóstica em inquéritos epidemiológicos de HIV/síndrome da imunodeficiência adquirida (Aids).

Unitermos: anticorpos anti-HIV; western blotting; estudos de validação; teste em amostras de sangue seco; sorodiagnóstico da Aids.

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