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Evaluation of the "shelf life" of nitrocellulose membranes immobilized with *Paracoccidioides brasiliensis* antigen for performing the immunodiagnosis of paracoccidioidomycosis

Avaliação do "tempo de prateleira" de membranas de nitrocelulose imobilizadas com antígeno de *Paracoccidioides brasiliensis* para o imunodiagnóstico da paracoccidioidomicose

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ABSTRACT

The immunoblotting reaction for performing the paracoccidioidomycosis (PCM) immunodiagnosis is an in-house methodology; and being a laborious task involving two previous steps, SDS-PAGE and Western blot, we evaluated the shelf life of nitrocellulose membranes containing the immobilized *P. brasiliensis* antigens, stored at -20 ºC for 7, 15, 30, 45, 60 and 90 days. Twenty-eight serum samples were analyzed on two nitrocellulose membranes groups: (a) membranes previously blocked with PBS-5 % non-fat dry milk and (b) the priory non-blocked membranes. No difference was detected in the reactivity pattern in serum samples evaluated in the both membrane groups, especially for those stored for 7, 15, 30, 45 and 60 days. It might be emphasized that a good stability of *P. brasiliensis* antigens, immobilized on the nitrocellulose membranes, enable them to be stored up to 60 days at -20 ºC. This finding contributes to the rapid diagnosis of PCM, and for sending them to other laboratories without adequate infrastructure for carrying out the steps that precede the immunodetection as the antigen production, SDS-PAGE and Western blot techniques. This scheme contributes substantially to improve the quality of PCM serodiagnosis, as it provides reproducible results in the units of the Laboratory Network.

Keywords. *Paracoccidioides brasiliensis*, paracoccidioidomycosis, immunoblotting, serologic tests.

RESUMO

Considerando-se que o *immunoblotting* para o imunodiagnóstico da paracoccidioidomicose (PCM) é uma metodologia *in house* e laboriosa envolvendo duas etapas iniciais, SDS-PAGE e *Western blot*, neste estudo foi avaliado o tempo de prateleira das membranas de nitrocelulose sensibilizadas com antígeno de *P. brasiliensis*, armazenadas a -20 ºC durante 7, 15, 30, 45, 60 e 90 dias. Vinte e oito amostras de soro foram analisadas em dois grupos de membranas de nitrocelulose (membranas previamente bloqueadas com PBS-leite 5 % e as não-bloqueadas). Não houve diferença no padrão de reatividade quando os soros foram avaliados frente a ambos os grupos, especialmente para membranas armazenadas por 7, 15, 30, 45 e 60 dias. A boa estabilidade do antígeno utilizado para sensibilizar as membranas fez com que estas pudessem ser armazenadas a -20 °C até 60 dias. Estas características contribuem para efetuar o diagnóstico rápido da PCM, bem como as perspectivas dessas membranas sensibilizadas serem encaminhadas para os laboratórios, que não possuam infraestrutura necessária para executar as etapas que antecedem a realização de *immunoblotting*, como a produção de antígeno, as técnicas de SDS PAGE e *Western blot*. Este procedimento contribui substancialmente para melhorar o diagnóstico sorológico da PCM, pois poderá fornecer resultados reprodutíveis nas unidades componentes da Rede de Laboratórios.

Palavras-chave. *Paracoccidioides brasiliensis*, paracoccidioidomicose, immunoblotting, testes sorológicos.

INTRODUCTION

Paracoccidioidomycosis (PCM) is the most important systemic mycosis of Latin America, especially in Brazil, caused by the thermally dimorphic fungi *P. brasiliensis* complex and *P. lutzii*[1-5] In endemic areas, the estimated incidence is approximately one to three cases per 100.000 inhabitants per year[6]. In Brazil the real incidence of PCM is still underestimated because according to the Brazilian regulations, the mandatory notification of this disease has not been required[7]. The definitive diagnosis of PCM is usually based on the demonstration of multibudding yeast cells in different biological specimens and/or the isolation of the fungus by culturing; however, the former shows low sensitivity, and the latter is time-consuming[8,9]. Consequently, serological techniques are an important tool not only for disease diagnosis, but also for monitoring the patient response to treatment[8,10]. Circulating antibodies to *P. brasiliensis* can be detected by serological assays such as double immunodiffusion (ID), counterimmunoelectrophoresis, ELISA, immunoblotting (IB)/Western blott (WB), latex agglutination and dot-blot[10-18]. Despite the availabilty of these serological assays, none of them has been providing high rates of intrinsic parameters such as sensitivity and specificity for an accurate diagnosis[11]. Among these methodologies, ID test has been routinely used by clinical laboratories due to its easy procedure, the low cost involved in its execution as well as its high specificity (about 100 %) and sensitivity (65 %–90 %)[9-11]. However, ID false negative results in PCM patients with active infection have been reported[19]. According to Do Valle et al.[20] several causes for these false negative reactions might be suggested: a) the prozone effect due to the excess of antigens owing to the extensive dissemination of disease; b) the formation of immune complexes with occluded epitopes; c) the presence of asymmetric antibodies which inhibit the secondary binding in precipitation reactions; and d) the occurrence of antibody contents below the method sensitivity. Our group has demonstrated that the use of IB assay significantly improves the sensitivity of PCM immunodiagnosis[13,15,16]. In addition, the IB is able to determine the reactivity of specific antigenic fractions, such as gp43 and gp70. These results demonstrate that ID and IB assays show to be complementary for the presumptive diagnosis and prognosis of disease, and possibly for early detection of relapses. These findings suggest that at least two serological tests for specific antibody detection should be used in cases of questionable diagnosis. In a general way, the shelf life is defined as the maximum period of time recommended for storing products, during which the defined quality of a specified proportion of the goods remains acceptable under expected (or specified) conditions of distribution, storage and display[21]. It applies to foods, beverages, pharmaceutical drugs, biological or chemicals reagents, diagnostic kits and many other perishable items[21]. It is known that clinical laboratories have increasingly sought to provide a "rapid response" not only aiming at the diagnosis, but also the surveillance of numerous diseases of public health importance. Considering the statement above cited as well as the fact that the IB reaction for PCM immunodiagnosis happens to be an in-house methodology, and being a laborious technique involving two previous steps, SDS-PAGE and WB, this study aimed at evaluating the shelf life of nitrocellulose membranes containing immobilized *Paracoccidioides brasiliensis* antigen.

MATERIAL AND METHODS

Sera Samples

Twenty-eight serum samples were evaluated in this study. Fourteen were randomly selected, and collected from patients with clinical suspicion of paracoccidioidomycosis to perform the presumptive diagnosis, besides twelve reagent serum samples and two samples showing negative reactivity to *P. brasiliensis* antigen in double ID and IB assays.

Consent

Informed consent was obtained from the patients for publishing this report. This research project was submitted and approved by the Ethical Committee for Research with human subjects of Adolfo Lutz Institute (CEPIAL nº 31/09).

Paracoccidioides brasiliensis Antigens

The employed antigenic preparation was a 20-day culture filtrate (Ag C) obtained from the yeast phase of *P. brasiliensis* according to Garcia et al[22] and modified by Silva et al[13]. Briefly, the fungus was cultured in NGTA (Neopeptone, Glucose, Thiamine, Asparagine) liquid medium for 20 days at 36 ºC under shaking. After the incubation time, the culture was treated with borate-thimerosal solution (1:5,000), filtered, divided into small volumes and stored at 4 ºC until use.

SDS-PAGE and Immunoblotting Assay

For performing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), *P. brasiliensis* antigens were diluted in buffer containing 62 mM Tris-HCl (pH 6.8), 2 % (wt/vol) SDS, 50 mM 2-mercaptoethanol, 10 % glycerol, and 0.01 % bromophenol blue, then boiled for 3 min and centrifuged before being applied to gels. Then, the antigens were subjected to electrophoresis (20 mA at room temperature) on a 10 % discontinuous SDS buffer system[23] in a Mini-protean II, Electrophoresis Cell (Bio-Rad Laboratories, Richmond, CA, USA). The molecular mass was determined by using 6.5–175 kD pre-stained standard protein marker (New England BioLabs). WB assays were carried out as previously described[24]. Proteins from SDS-PAGE were electrotransferred onto 0.20 µm nitrocellulose membranes (Sigma Chemical Co. St. Louis, Mo, USA) in a Mini Trans-Blot Cell (Bio-Rad Laboratories, Richmond, CA, USA), with 25 mM Tris, 192 mM glycine, pH 8.3, and methanol 20 % (v:v). The IB protocols were performed according to Passos[16]. Thus, the nitrocellulose membranes containing electrophoresed antigens were blocked with 5 % non-fat dry milk in PBS (PBS-5 % L) for sixty min at room temperature (25 ºC). The membranes were incubated for 120 min at room temperature with human sera diluted at 1:100 in PBS containing 3 % non-fat dry milk (PBS-3 % L). After that, they were washed six times with PBS containing 0,1 % (w/v) of Tween 20 (PBS-T) and developed with peroxidase-conjugated goat anti-human IgG antibody (Sigma Chemical Co. St. Louis, Mo, USA) diluted at 1:3,000 in PBS-3 % L, and incubated for 90 minutes under stirring at room temperature (25 ºC), protected from light. The membranes were washed again six times for 10 minutes each in PBS-T. The reactions were revealed by employing a solution of 4-chloro-1 naftol (Sigma-Aldrich Co. St. Louis, MO, USA), and the blocking was made by successive washes in distilled water.

Evaluation of the shelf life of nitrocellulose membranes

Nitrocellulose membranes containing immobilized *P. brasiliensis* antigen were divided into two groups: a) membrane blocked with PBS-5 % L was incubated for 60 min in shaker platform at room temperature, and then washed twice with PBS pH 7.4; b) priory non-blocked membranes. The membranes were then wrapped in plastic film and aluminum foil, and stored at -20 °C for 7, 15, 30, 45, 60 and 90 days. At the time previously established, the membranes were removed from the freezer to perform the IB assays.

RESULTS

Concerning the stability of nitrocellulose membranes, no difference in the reactivity pattern was found when the serum samples were evaluated against the both groups, i.e., the membranes previously blocked with PBS-5 % non-fat dry milk for 60 min at room temperature, and those kept immediately after the transfer process, especially for membranes stored for 7, 15, 30, 45 and 60 days.

Although, the impaired reactivity pattern was observed in those membranes stored for a period exceeding 60 days. In the membranes evaluated after 90 days of storage, the samples showed lower reactivity and poor intensity pattern or absence of reactivity when compared to the membranes of other storage periods. Furthermore, these results indicated that the nitrocellulose embranes containing *P. brasiliensis* immobilized antigen, stored at -20 ºC in two distinct conditions, did not alter the antigenicity. These findings indicated that regardless of the membranes storage condition, the antigen remains stable and capable to recognize the species-specific antibodies, especially the anti-glycoproteins of 43 and 70 kDa, present in sera from patients with active disease or with clinical suspicion of paracoccidioidomycosis (**Figure**).

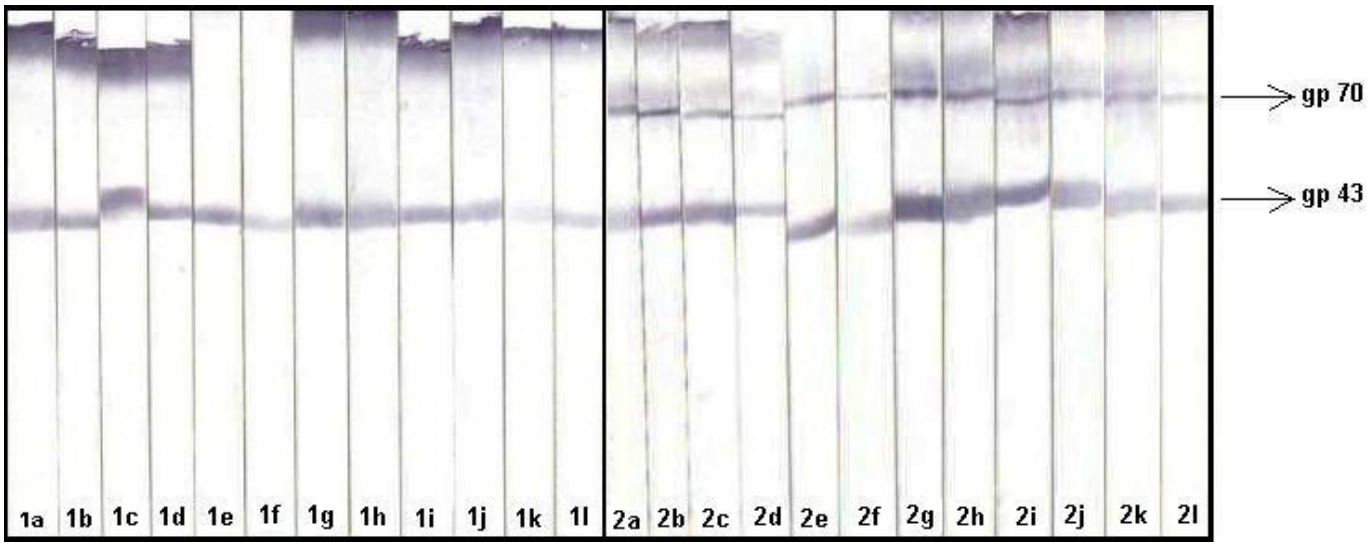

Figure. Stability of the nitrocelulose membranes containing culture filtrate sample of 113 *Paracoccidioides brasiliensis***,** stored at -20 °C**,** and analyzed on immunoblotting assay. Membranes were divided into two groups: (1) membrane blocked with PBS-5 % L (a,c,e,g,i,k) and (2) priory non-blocked membranes (b,d,f,h,j,l), and stored for 7 (a,b), 15 (c,d), 30 (e,f) , 45 (g,h), 60 (i,j) and 90 (k,l) days

DISCUSSION

Serological techniques are usually simpler than culture methodology, being very useful for carrying out the presumptive diagnosis and for following-up the patients infected with *Paracoccidioides brasiliensis.* Among them, double ID assay remains as the main serological test for paracoccidioidomycosis[8-11]. Passos et al[15] evaluated the performance of ID and IB assays in the serological diagnosis of PCM, showing that the agreement analysis between these techniques performed by Cohen kappa index was considered good (k= 0.76). Both methods demonstrated high efficiency (>90 %), and IB showed a better ability to discriminate the positive samples, when the diagnosis was not feasible by ID, corroborating the results observed by Silva et al[13]. These findings demonstrated that ID and IB assays showed to be complementary to the presumptive diagnosis, the prognosis of disease**,** and being possible to perform the early relapses detection, suggesting that at least two serological tests for antibody detection should be employed in cases of questionable diagnosis. Although, despite the advantages of this approach, it should be noted that for performing the immunodiagnosis of paracoccidioidomycosis, no commercial IB or WB kits have been available until now. Thus, these two methods have been carried out thoroughly as homemade and as hand-crafted techniques at mycology-serology laboratories**.** Both techniques can be classified as a complex and time-consuming procedure, as they involve several specific steps, before performing the immunodetection. These techniques require some approaches as the separation of proteins or of *P. brasiliensis* antigenic fractions by SDS-PAGE, followed by electrophoretic transfer of the fungal components to the nitrocellulose membrane. A high level of stability is essential for any biological pattern, and also it is desirable to the majority of organic products[21]**.** For this reason, it is relevant to evaluate the shelf life of the stored membranes to investigate whether they maintain the standard efficiency/immunoreactivity with the same quality of those newly produced. Thus, the use of serological tests which satisfy the characteristics as the rapid and easy implementation**,** and especially with good sensitivity, specificity, positive and negative predictive values, and accuracy has become increasingly frequent. In paracoccidioidomycosis field, this is the first study that addresses the

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stability or shelf life of nitrocellulose membranes containing immobilized *P. brasiliensis* antigen. In the present study, an in-house immunoblotting assay was standardized, in which the nitrocellulose membranes containing *P. brasiliensis* immobilized antigens were employed. It was demonstrated that their storage at -20º C in two distinct conditions did not interfere in their antigenic characteristics. These findings indicate that regardless the nitrocellulose membranes storage condition**,** the antigen remains stable and effective to recognize the species-specific antibodies, especially the anti-glycoproteins of 43 and 70 kDa present in serum samples collected from patients with active disease or with clinical suspicion of paracoccidioidomycosis. In conclusion**,** it can be stated that the high sensitivity, the relative specificity associated with the good stability of nitrocellulose membranes strengthen the fact that the IB assay significantly improves the sensitivity of PCM immunodiagnosis**.** Also, it is suggested that at least two serological tests for specific antibody detection should be took over in the cases of questionable diagnosis found in double ID and ELISA**.** It should also be noted that the long durability of antigens immobilized into nitrocellulose membrane provides perspective for shipping them to other laboratories without adequate infrastructure for carrying out all of the steps for performing these immune-detection techniques**,** as the production of antigen, SDS-PAGE and WB techniques. Thus**,** these findings will contribute substantially to improve the serodiagnosis of PCM, and to provide reproducible results in the units of the Laboratory Network.

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