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Multiparametric assay of screening for the diagnosis of mycoses of interest in Public Health: standardization of methodology

Ensaio multiparamétrico de triagem para diagnóstico de micoses de interesse em Saúde Pública: padronização da metodologia

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ABSTRACT

The standardization and validation of a multiplex assay requires the combination of important parameters such as sensitivity and specificity, acceptable levels of performance, robustness, and reproducibility. We standardized a multiparametric Dot-blot aimed at the serological screening of paracoccidioidomycosis, histoplasmosis, and aspergillosis. A total of 148 serum were evaluated: 10 from healthy subjects, 36 from patients with paracoccidioidomycosis, 62 from patients with histoplasmosis, and 40 from patients with aspergillosis. It was found that the multiparametric Dot-blot showed a high percentage of cross-reactivity. However, when evaluated individually, in the serological screening of histoplasmosis, a good performance was observed when compared to the double immunodiffusion assay, considered the gold standard test, with 100% co-positivity and 83.3% co-negativity. The performance of serological screening for aspergillosis was not satisfactory when compared to double immunodiffusion, showing 71.4% co-positivity and 100% co-negativity. The evaluation of the stability of nitrocellulose membranes showed that membranes sensitized with *H. capsulatum* antigen remained stable for 90 days and those sensitized with *A. fumigatus* antigen for 30 days. We conclude that the use of crude antigens was not suitable for the standardization of the multiparametric Dot-blot assay, due to the high cross-reactivity, and that further tests should be performed with purified proteins.

Keywords. Paracoccidioidomycosis, Histoplasmosis, Aspergillosis, Immunologic Tests, Dot-blot.

RESUMO

A padronização e validação de um ensaio multiplex requer a combinação de parâmetros importantes, como sensibilidade e especificidade, níveis aceitáveis de desempenho, robustez e reprodutibilidade. Este trabalho padronizou um Dot-blot multiparamétrico visando a triagem sorológica da paracoccidioidomicose, histoplasmose e aspergilose. Foram avaliadas 148 amostras de soro: 10 de indivíduos saudáveis, 36 de pacientes com paracoccidioidomicose, 62 de pacientes com histoplasmose e 40 de pacientes com aspergilose. Verificou-se que o Dot-blot multiparamétrico apresentou elevado percentual de reatividade cruzada. Entretanto, quando avaliado individualmente, na triagem sorológica da histoplasmose observou-se bom desempenho quando comparado ao ensaio de imunodifusão dupla, considerado o teste padrão ouro, com 100% de co-positividade e 83,3% de co-negatividade. O desempenho da triagem sorológica da aspergilose não foi satisfatório quando comparado a imunodifusão dupla, apresentando 71,4% de co-positividade e 100% de co-negatividade. A avaliação da estabilidade das membranas de nitrocelulose mostrou que membranas sensibilizadas com antígeno de *H. capsulatum* permaneceram estáveis por 90 dias e as sensibilizadas com antígeno de *A. fumigatus*, por 30 dias. Concluímos que o uso de antígenos brutos não foi adequado para a padronização do ensaio de Dot-blot multiparamétrico, devido ao alto índice de reatividade cruzada, e que novos testes devem ser realizados com proteínas purificadas.

Palavras-chave. Paracoccidioidomicose, Histoplasmose, Aspergilose, Testes Immunológicos, Dot-blot.

INTRODUCTION

Systemic fungal infections have increased dramatically in incidence, prevalence, and severity over the last few decades, in concert with the number of patients living for extended periods with significant immune dysfunction. AIDS, cancer chemotherapy, and organ transplantation have all contributed to this rise, as has the widespread use of the antibiotics[1]. The use of serological assays is extremely important in the diagnosis of infectious processes caused by endemic or opportunistic fungi, since the demonstration in direct examination as well as the isolation and identification of these fungi often has negative results, especially in cases of self-limited diseases[2-6].

Among the different serologic assays, the Double Immunodiffusion (DI) is the "gold standard" methodology used for the diagnosis of paracoccidioidomycosis, histoplasmosis and aspergillosis. The DI assay has high specificity; however, depending on the *P. brasiliensis* antigen used, the sensitivity can range from 65 to 100%[7]; 70 to 95% for *H. capsulatum*[3]; and 89.3 to 100% for *A. fumigatuss*[5,6]. Although the DI assay has advantages in cost and feasibility, there is a need to implement in serological routine a more rapid and sensitive test that contributes with the disease screeening in order to initiate appropriate therapy, prevention of further damage and monitor fungal dissemination to other organs[2,4-7]. For the detection of many protozoan, viral and fungus diseases, Dot-blot has been widely accepted as a rapid, versatile and simple test based on the principle of enzyme immunoassays[8,9]. The greatest advance in the applicability of the Dot-blot methodology is described for the immunodiagnosis of paracoccidioidomycosis[9-13], followed by aspergillosis[14-16]; however there are no studies in the literature that address the use of this assay in relation to the diagnosis of histoplasmosis. Based on the previous experience of the Laboratório de Imunodiagnóstico das Micoses, with the Dot-blot assay in paracoccidioidomycosis, it was decided to standardize the Dot-blot aiming at the simultaneous diagnosis of paracoccidioidomycosis, histoplasmosis and aspergillosis.

METHODS

Serum samples

A total of 148 serum samples were evaluated, including 36 samples from patients with paracoccidioidomycosis, 62 from patients with histoplasmosis and 40 from patients with aspergillosis. Ten serum samples from healthy individuals (normal human serum [NHS]) were used as negative control and anti-*P. brasiliensis*, anti-*P. lutzii*, anti-*H. capsulatum* and anti-*A. fumigatus* polyclonal antibodies, obtained from rabbits, were used as a positive control.

Ethics consideration

This research was conducted within the standards required by Resolution 466 of December 12, 2012, of the Ministério da Saúde – Conselho Nacional da Saúde, and it was approved by the Comitê de Ética em Pesquisa of Adolfo Lutz Institute (CEPIAL nº. 13673313.1.0000.0059).

P. brasiliensis antigen

The antigen used was a 20-day culture filtrate (Ag CF) obtained, according to Silva[17], from

the yeast phase of 113 *P. brasiliensis* strain. The fungi were cultured in NGTA 3% (w/v) neopeptone, 1.8% (w/v) glucose, 0.009% (w/v) asparagine and 0.125% (w/v) thiamine liquid medium for 20 days at 36 °C with shaking. After incubation time, the cultures were treated with an aqueous borate-thimerosal solution (1:5,000) (Sigma Chemical Co., St. Louis, MO, USA), and left to stand for 96 hours at 4 °C. After this, the supernatants were filtered through Whatman® nº. 1 paper (Whatman, Brentford, UK), divided into small volumes, and stored at 4 °C until use. Protein contents were subsequently assessed by the Bradford method[18].

H. capsulatum antigen

The *H. capsulatum* antigen was obtained, according to Freitas[19] and Freitas et al[20]. Mycelial cells of 200 *H. capsulatum* strains were grown in solid Sabouraud-dextrose (Difco Laboratories, Detroit, MI, USA) medium at 27 °C during 33 days. After incubation time, the cultures were treated with an aqueous solution of thimerosal 1:5,000 (Sigma Chemical Co., St. Louis, MO, USA) and left to stand for 24 hours at room temperature. After this, the supernatants were filtered through Whatman® nº. 1 paper (Whatman, Brentford, UK), constituting the preparation of *H. capsulatum* antigen. Antigens were concentrated 10 to 20-fold by a lyophilization procedure. Protein contents were subsequently quantified by the Bradford method[18].

A. fumigatus antigen

Seven-day mycelial cells from 734, 736 and 727 of *A. fumigatus* strains were inoculated into 500 mL Erlenmeyer flasks containing 250 mL of Sabouraud dextrose broth (Difco Laboratories, Detroit, MI, USA), and incubated for 30 days at 27 °C, in a stationary phase. After this period, the cultures were inactivated by the addition of 1:5,000 thimerosal (Sigma Chemical Co., St. Louis, MO, USA) solution and left to rest for five days at 4 °C. Then they were filtered on Whatman® paper nº. 3 (Whatman, Brentford, UK) and the supernatants were concentrated by lyophilization (Edward's – Super Modulyo) and stored at -20 °C until the moment of use. Protein contents were subsequently quantified by the Bradford method[18].

Dot-blot assay

The methodology was in PBS (pH 7.4) containing 5% non-fat dry milk (PBS-L 5%), under constant shaking at room temperature. These adsorbed and blocked membranes were stored at room temperature until use. For antibody detection, the membranes were placed on plates containing channels and then incubated for 2h with 1 mL dilutions of either 1:100 of the test sera (individual sera from patients with paracoccidioidomycosis, histoplasmosis, aspergillosis and sera from healthy individuals as well as anti-fungal polyclonal antibodies) in PBS containing 3% non-fat dry milk (PBS-L 3%), under constant shaking at room temperature. After three washes with 1 mL per well of 0.1% Tween-20 in PBS (PBS-T 0.1%), the membranes were immersed in a 1:2,000 dilutions of secondary antibody: goat anti-humam IgG immunoglobulin conjugated to peroxidase (Sigma-Aldrich Co., St. Louis, MO, USA) in PBS-L 3% for 90 minutes, at room temperature. After this incubation time the membranes were washed again. Then, the membranes were immersed in a fresh mixture prepared using 15 mg of 4-chloro-1-naphtol (Sigma-Aldrich Co., St. Louis, MO, USA) diluted in 5mL of absolute methanol, 30 µL of 30-vol hydrogen peroxide and 20 mL Tris-HCl 0,5 M pH 6.8. The reaction was stopped by washing with distilled water.

Nitrocellulose membranes were dried on filter paper and the development of blue dots was considered evidence of a positive result.

Three researchers with expertise in enzyme-linked immunosorbent assays such as immunoblotting and ELISA performed the analysis of a serum anonymous sample. The samples were identified with sequential numbering to avoid identification of cases and controls by analysts. The DB assays were then performed on different days, including all the steps of the method. The test was carried out in duplicate to evaluate the intra- and inter-related content.

Analysis of the membranes stability

Nitrocellulose membranes previously sensitized with the *P. brasiliensis*, *H. capsulatum* and *A. fumigatus* antigens, were maintained at room temperature and evaluated progressively for their antigenic reactivity over the time periods of 7, 15, 30, 45, 60 and 90 days.

Double immunodifusion assay

The reactions were performed according to the modified Ouchterlony's method[22]. Glass slides were covered with 3.0 mL of a gel composed of 1% agarose type II medium (Sigma Chemical Co., MO, USA) in a buffered saline solution pH 6.9 containing 0.4% sodium citrate and 7.5% glycine. Antigen (12 µL) was placed in the central well, while control and patient sera (12 µL) were put in the surrounding wells. The slides were incubated in a humid chamber at room temperature for 48 hours. Then, they were washed with saline solution with several changes over a 24-hour period. Gels were dried and stained in 0.4% Coomassie brilliant blue R-250® (Sigma Chemical Co., MO, USA) in an ethanol-acetic acid-water mixture as solvent.

RESULTS

The analysis of the results for Dot-blot for the immunodiagnosis of histoplasmosis revealed 100% co-positivity and 83.3% co-specificity and, for *A. fumigatus*, the intrinsic parameter calculations were 71.4% and 100% for co-positivity and co-specificity, respectively.

The shelf life or stability of nitrocellulose membranes sensitized with *H. capsulatum* and *A. fumigatus* antigens showed a different pattern from that previously obtained for membranes sensitized with *P. brasiliensis*[13,21]. For histoplasmosis the membranes revealed stability for 90 days and for aspergillosis the membranes remained stable for 30 days.

The analysis of the results of the multiparametric Dot-blot revealed the occurrence of cross-reactivity both of the species-specific polyclonal antibodies and of the serum samples with the different antigenic preparations immobilized on the nitrocellulose membrane. A high cross-reactivity index was observed when serum samples from patients with paracoccidioidomycosis were evaluated: 82% of the samples also showed reactivity against the *H. capsulatum* antigen and 48.4% against the *A. fumigatus* antigen. Of the total serum samples from patients with histoplasmosis, 55% reacted against the antigen of *P. brasiliensis* and 27% against the antigen of *A. fumigatus* and of the total of serum samples from patients with aspergillosis, 52% also showed reactivity towards the antigen. *P. brasiliensis* and 64% against *H. capsulatum* antigen as presented in **Figure**.

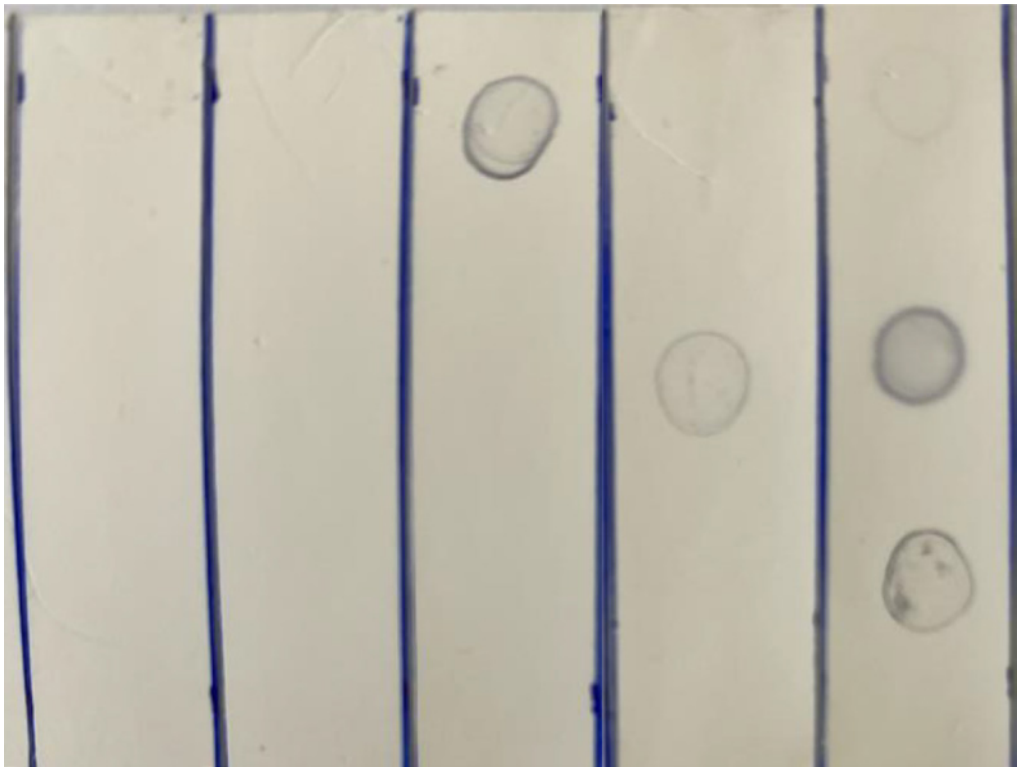

Figure. Cross-reactivity *A. fumigatus*' polyclonal species-specific anti-antigen against *P. brasiliensis* and *H. capsulatum* antigens, where Ag Pb113 is the antigen of *P. brasiliensis*, sample 113; Ag Hc200 is an antigen of *H. capsulatum*, isolated 200; Ag Af356+727 is the antigen of *A. fumigatus*, pool of samples 356 and 727; IS means healthy individual; C+ Pb means tape incubated with the polyclonal anti-antigen of *P. brasiliensis*; C+ Hc means tape incubated with the anti-antigen of *H. capsulatum* and C+ Af means tape incubated with the anti-antigen of *A. fumigatus*

DISCUSSION AND CONCLUSION

Given the excellent experience obtained with the standardization and validation of the Dot-blot methodology for the immunodiagnosis of paracoccidioidomycosis and seeking new alternatives that could contribute to the quick diagnosis of the infectious processes caused by *H. capsulatum* and *A. fumigatus*, for the development of this study it was proposed to standardize the multiparametric Dot-blot assay with the objective of simultaneously evaluating and discriminating positive or highly suspicious cases for the three conditions, that are, paracoccidioidomycosis, histoplasmosis and aspergillosis.

The standardization of the multiparametric assay preceded the individual assessment of Dot-blot for histoplasmosis and aspergillosis. Given the high rate of co-positivity, it can be suggested that the Dot-blot methodology aimed at the immunodiagnosis of histoplasmosis can be used as a serological screening test, helping as observed in the Dot-blot for *P. brasiliensis*, for the quickly release of discarded cases, allowing that the diagnostic hypothesis initially formulated to be reviewed. On the other hand, the high co-specificity index suggests that Dot-blot for the immunodiagnosis of aspergillosis can be used as a test for diagnosis, since it discriminated against all truly positive sera.

The high rate of cross-reactivity observed in the multiparametric Dot-blot was not new, since this phenomenon has been frequently reported in the immunodiagnosis of mycoses by several authors. There

are several hypotheses for this. One of them, perhaps the most important, focuses on the composition of the cell wall of fungal species. The three main components of the cell wall, shared by most clinically important fungal species, are: β-glucans (which are polymers of glycose), especially β-(1,3)-glucans; chitin (which is a polymer of N-acetylglucosamine) and mananas (which are chains of several hundred mannose molecules that are added to fungal proteins through N or O linkages)[23-25]. Connolly et al[26], in an attempt to develop an quantitative enzyme immunoassay for the diagnosis of histoplasmosis demonstrated cross-reactivity in 80% of samples from patients with paracoccidioidomycosis or pneumocystosis, in 60% of those with coccidioidomycosis, and almost 10% of those with aspergillosis.

Other hypotheses that can explain the occurrence of reactivity crossed are: the overlapping of the ecological niche of different fungal species; the composition of the cell wall; the ubiquity of antigenic determinants; the presence of heterophile antibodies and the great antigenic similarity between dimorphic fungi[27-32]. Sato et al[27] evaluated the performance of the Dot-ELISA assay aiming the investigation of circulating anti-*B. dermatitidis* antibodies against 21 serum samples from patients with blastomycosis, obtaining a sensitivity of 76.2%.

The occurrence of cross-reactivity was observed against antigenic preparations of *H. capsulatum* and *Coccidioides immitis*. According to the authors, as the endemic of blastomycosis and histoplasmosis overlap one cannot be completely sure that anti-*Histoplasma* antibodies are absent in those patients with diagnosed blastomycosis. Two works developed in the Laboratório de Imunodiagnóstico das Micoses, support and corroborate the hypothesis that the similarity antigenicity or sharing of antigenic determinants may be involved in cross-reactivity. Beraldo[30] confirmed the existence of cross-reactivity between *P. brasiliensis* and *P. lutzii* by observing that 91.66% of serum samples from patients with confirmed paracoccidioidomycosis, residing in a non-endemic region for *P. lutzii*, and with serological reactivity for *P. brasiliensis*, showed reactivity against *P. lutzii* antigenic preparations. It was also observed that 76.19% of serum samples from patients with paracoccidioidomycosis, residing in the state of Rondônia, Brazil, a potentially endemic region for *P. lutzii*, also showed serological reactivity against both antigenic preparations. In the same study, the evaluation of the electrophoretic profile of *P. lutzii* and *P. brasiliensis* antigens reveals that both species present in their antigenic constitution fractions that are common to each other.

Buccheri et al[31] confirmed the antigenic complexity present in the genus *Paracoccidioides* and warned of the need for a careful interpretation of the results generated. In this work, the authors used the immunoblotting assay to verify the pattern of recognition from a biological sample obtained from a patient with no epidemiological link to *P. lutzii*, but with serological reactivity, titer of 1:256, by double immunodiffusion assay. Thus, the sample was evaluated by immunoblotting, against the culture filtrate obtained from the *P. brasiliensis* isolate 113 and against the cell free antigen of *P. lutzii*, revealing strong recognition of the 43 and 70 kDa fractions of *P. brasiliensis* and the recognition of multiple fractions, between 26 to 115 kDa, of *P. lutzii*. Using the semi Nested PCR assay, the patient sequence was found to show 98% identity when compared to the *P. brasiliensis* gp43 sequences available from GenBank®.

The use of purified or recombinant antigens was discarded for the development of this work for two reasons. The first is that this strategy makes the method more expensive and does not match the philosophy of the Laboratório de Imunodiagnóstico das Micoses, that is, the development of low-cost products that can be used by laboratories without so many technological and human resources. The second is that different studies have shown the existence of cross-reactivity even when using recombinant antigens. The most recent, published by Peron et al[32] aiming at the diagnosis of paracoccidioidomycosis,

showed that the use of rHSP60 did not prove to be a good diagnostic option due to the high index of cross reactions observed.

This research project was idealized thinking mainly about developing a diagnostic platform that could serve as a tool for screening or even for the rapid and accurate diagnosis of paracoccidioidomycosis, histoplasmosis and aspergillosis. The expectation was that the multiparametric Dot-blot could be validated and made available to the Public Health system, not only at the Adolfo Lutz Institute, but that it could be shared and implemented in more distant laboratories and with less installed capacity in both it concerns the technological park as well as in relation to human resources when compared to the laboratories considered to be reference or universities that are strategically and geographically located in large centers.

It was also expected that the multiparametric Dot-blot could be used in the field, helping to carry out seroepidemiological surveys, as Brazil lacks information on the prevalence of mycoses. The expectation was that Dot-blot would be developed without the need to use more refined and complex technologies, such as, for example, the use of purified or recombinant antigens and even chemical treatment of the antigens aiming at their deglycosylation, a procedure that oxidizes the glycidic part of the antigens contributing to the reduction of cross-reactivity, as these procedures increase the final cost of the product.

Although this study did not reach its main objective, that is, the standardization of a platform that would allow the simultaneous assessment of circulating antibodies against *Paracoccidioides* spp., *H. capsulatum* and *A. fumigatus*, we are certain of the need to conduct other studies that further investigate the serological approach for the diagnosis of these mycoses.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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AUTHORS' CONTRIBUTIONS

Camila Mika Kamikawa conducted laboratory evaluations and drafted the first version of the article. Adriana Pardini Vicentini made critical reviosion of the text. All authors read and approved the final manuscript.

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PRESENTATION NOTE

The partial results of this article integrate the doctoral thesis by Camila Mika Kamikawa, entitled "Standardization of the Dot-blot methodology for the multiparametric diagnosis of mycoses of interest in Public Health", defended in 2020, in the Programa de Pós-Graduação em Ciências of the Coordenadoria de Controle de Doenças da Secretaria de Estado da Saúde de São Paulo.

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