

Dot-Blot Methodology for Rapid Diagnosis of Paracoccidioidomycosis Caused by *Paracoccidioides brasiliensis*

Kamikawa CK^{1,2} and Vicentini AP^{1,2*}

¹Mycoses Immunodiagnostic Laboratory, Immunology Center, Adolfo Lutz Institute, São Paulo, Brazil

²Postgraduate Program in Sciences, Disease Control Coordination, State Secretariat of Health of São Paulo, São Paulo, São Paulo, Brazil

*Corresponding author: Adriana Pardini Vicentini, Laboratório de Imunodiagnóstico das Micoses, Centro de Imunologia, Instituto Adolfo Lutz, Av. Dr. Arnaldo, 351, 11º andar, sala 1104, São Paulo, SP, 01246-902, Brazil, Tel: +55113068 2900; Email: apardini@ial.sp.gov.br

Received date: October 12, 2015; Accepted date: December 22, 2015; Published date: December 28, 2015

Copyright: © 2015 Kamikawa, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

Paracoccidioidomycosis (PCM), a mycosis which clinical diagnosis can be confused with other infectious diseases like leishmaniasis and tuberculosis, presents significant rates of mortality, estimated between 2-23% in severe cases, reaching 30% when associated with AIDS [1], and considered to lead mortality among mycosis in Brazil, with an average of 111.5 deaths/year, according to the Mortality Information System of the Ministry of Health [2]. Given the above, an early and accurate diagnosis of this infectious disease is very important because it allows the establishment of appropriate antifungal therapy, reduction of unnecessary use of toxic drugs; decreasing the use of empiric therapy, thereby minimizing the emergence of multidrug-resistant fungal strains. In addition, the technique used to immunodiagnostic of PCM has to combine sensitivity and specificity, so that the predictive value is high and reproducible. In this study, Dot-blot (DB) method was found to be an extremely promising tool as serologic screening technique, because of its high sensitivity, as well as a rapid diagnostic test, given the release of true negative results in less time (one day) compared to the double immunodiffusion assay in agarose gel (DI, seven days), considered the gold standard serological method and, therefore, of great diagnostic value, particularly for the disposal of clinical suspicion for the this mycosis. Furthermore, Dot-blot method shows the prospect of being transferred to laboratories of public service including those with fewer resources of facilities such as photometers, plate washers and others, or even be used directly in the field, with an excellent shelf life validity, ie membranes coated with antigen can be used for testing, without changes in the pattern of reactivity among laboratories and present reliable values of sensitivity, specificity, predictive values, accuracy and a high correlation with the serological gold standard methodology, therefore to be used in routine diagnosis for Public Health.

Introduction

Paracoccidioidomycosis, a highly endemic mycosis in Brazil, is caused by fungal species of *Paracoccidioides* (*P. brasiliensis* and *P. lutzii*) [3,4]. The gold standard method for definitive diagnosis is based on budding yeast cells observation, by conducting direct examination, isolation and identification of the etiologic agent after culture of biological samples. However, isolation and culture are not easy and rapid to be performed, with often serial collection of samples, that contributes to the delay in the establishment of appropriate antifungal treatment; also adds to these observations, the low sensitivity of mycological methods [5]. Thus, serological techniques have been used as important diagnostic and prognostic tools, optimizing time for obtaining results [6]. The double immunodiffusion assay is widely used for PCM diagnosis due its high specificity and easily to perform with no automation, also allowing qualitative and semi-quantitative detection of species-specific antibodies, for monitoring the effectiveness of therapeutic treatment. Although DI is highly specific, its sensitivity is weak, so substantial number of false negative results have been reported, beside the long time period until the release of result (120 hours). The Dot-Blot technique has been successfully used in the diagnosis of many infectious and parasitic diseases, such as toxoplasmosis and visceral leishmaniasis, aiming to offer a faster response [7]. For the immunodiagnosis of PCM, the DB has shown to be considerable promise on monitoring patients during anti-fungal treatment and seroepidemiological surveys [8].

Objectives

The objective of this study was to optimize, standardize and validate the Dot-Blot test for rapid diagnosis of PCM, and to propose it as serological screening tool for patients with clinical suspicion for Paracoccidioidomycosis, by comparing to DI and evaluating the shelf life of coated membranes storage over the time.

Methodology

We evaluated 443 serum samples from patients, as follow: 143 grouped as control (23 samples from apparently healthy patients, 77 samples from patients with PCM confirmed by mycological and/or histopathological evaluations and 43 samples with other infectious diseases [tuberculosis, aspergillosis and/or histoplasmosis]) and 300 serum samples from patients with clinical suspicion of PCM, forwarded to the Immunodiagnostic of Mycoses Laboratory, Immunology Center, Instituto Adolfo Lutz. Tested exoantigens, were obtained following the methodology proposed by Garcia et al. and modified by Silva, using culture filtrates of sample 113 and B-339 strain of *P. brasiliensis*. Protein concentration was determined by Bradford method using NanoDrop® Spectrophotometer equipment ND-1000 (Thermo Fisher Scientific, DE, USA) [9-11]. Detection of anti-*Paracoccidioides brasiliensis* circulating antibodies was carried out using double immunodiffusion in agarose gel assay, proposed by Ouchterlony [12]. The Dot-Blot method was optimized based on the protocol described by Hawkes et al. and Pappas [7,13]. For the

serological test validation, we calculated intrinsic parameters of sensitivity, specificity, predictive values, prevalence, accuracy/efficiency, co-positivity (relative sensitivity) and co-negativity (relative specificity) according to Ferreira and Ávila. Analysis of concordance of results was evaluated by Kappa index Cohen obtained using table proposed by Landis and Koch [14,15]. Finally, for proportions comparison we used the chi-square test, performed by the Epi Info 6.1 program (Center for Disease Control and Prevention- <http://www.cdc.gov>) with $p \leq 0.05$ significance value.

Results

The Dot-blot method standardization showed better results using nitrocellulose membranes coated with antigen obtained from culture filtrate of B-339 isolated of *P. brasiliensis* and serum and conjugate diluted at 1:40 and 1:2000, respectively, both incubating in PBS-M 3% solution. Sensitivity, specificity, positive and negative predictive values and accuracy for DI and DB were calculated over results of 143 samples from control group, as follows: 98.2%, 75.5%, 72.7%, 98.4%, 40% and 84.6% for DI, 95.9%, 90%, 91%, 95.4%, 51% and 93% for DB, respectively. Performance of DB compared to DI standard method were evaluated using 300 samples from patients with clinical suspicion of PCM. Anti-*P. brasiliensis* circulating antibodies were detected in 34% of samples, while DB had detected these antibody in 47.3%. Discriminatory capacity of both methods suggested that DB test has a tendency to increase the reactivity pattern, as it was recognized by 40 (13.3%) more samples than DI. Copositivity (relative sensitivity) and co-negativity (relative specificity) values, using the DI assay as the standard serological test, were 68.3% and 96.8%, respectively, achieving an agreement level between them of $\kappa=0.66$, considered good.

Conclusion

A statistical analysis, demonstrated that Dot-blot technique was significantly more sensitive ($p=0.000067$) than Double Immunodiffusion methodology, proving to be an excellent tool for the serological screening of serum samples from patients with clinical suspicion and/or confirmed paracoccidioidomycosis. Dot-blot test showed lower specificity (95.4%) than that calculated for the reaction of double immunodiffusion (98.5%), confirming the applicability of the DI assay as diagnostic test. Dot-blot method showed low percentage of cross-reactivity when serum samples from patients with other lung infections such as histoplasmosis, tuberculosis and aspergillosis were evaluated. These results suggest a possible new algorithm for serological diagnosis of PCM, carrying out the serological screening with Dot-blot methodology instead of the indirect ELISA test.

Acknowledgment

We would like thank Marisa Ailin Hong for the careful review of the English.

Funding

This study was supported by Instituto Adolfo Lutz (CTC-IAL#117D/2012), Camila Mika Kamikawa's scholarship granted by CAPES. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Souza W (2010) Doenças Negligenciadas. Academia Brasileira de Ciências, Rio de Janeiro, pp. 43.
2. Ministério da Saúde [Internet Homepage]. Informações de Saúde [accessed in 08/04/2015].
3. Bocca AL, Amaral AC, Teixeira MM, Sato PK, Shikanai-Yasuda MA, et al. (2013) Paracoccidioidomycosis: eco-epidemiology, taxonomy and clinical and therapeutic issues. Future Microbiol 8: 1177-1191.
4. Teixeira Mde M, Theodoro RC, Oliveira FF, Machado GC, Hahn RC, et al. (2014) *Paracoccidioides lutzii* sp. nov.: biological and clinical implications. Med Mycol 52: 19-28.
5. Teles FR, Martins ML (2011) Laboratorial diagnosis of paracoccidioidomycosis and new insights for the future of fungal diagnosis. Talanta 85: 2254-2264.
6. de Camargo ZP (2008) Serology of paracoccidioidomycosis. Mycopathologia 165: 289-302.
7. Pappas MG (1988) Recent applications of the Dot-ELISA in immunoparasitology. Vet Parasitol 29: 105-129.
8. Tabora CP, Camargo ZP (1994) Diagnosis of paracoccidioidomycosis by dot immunobinding assay for antibody detection using the purified and specific antigen gp43. J Clin Microbiol 32: 554-556.
9. Garcia NM, Del Negro GM, Heins-Vaccari EM, de Melo NT, de Assis CM, et al. (1993) [*Paracoccidioides brasiliensis*, a new sample isolated from feces of a penguin (*Pygoscelis adeliae*)]. Rev Inst Med Trop Sao Paulo 35: 227-235.
10. Centro de Referência e Treinamento em DST/AIDS-São Paulo, Programa Estadual de DST/aids-São Paulo, Coordenadoria de Controle de Doenças, Secretaria de Estado da Saúde de São Paulo (2011) [Eliminating vertical transmission of HIV and syphilis in São Paulo State, Southeastern Brazil]. Rev Saude Publica 45: 812-815.
11. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of Protein-Dye Binding. Anal Biochem 72: 248-254.
12. Ouchterlony O (1949) Antigen-antibody reactions in gels. Acta Pathol Microbiol Scand 26: 507-515.
13. Hawkes R, Niday E, Gordon J (1982) A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem 119: 142-147.
14. Ferreira AW, Ávila SLM (2001) Diagnóstico laboratorial das principais doenças infecciosas e auto-imunes (2nd edn.) Guanabara Koogan, Rio de Janeiro, Brazil.
15. Landis JR, Koch GG (1977) The measurement of observer agreement for categorical data. Biometrics 33: 159-174.