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Paracoccidioidomycosis: Detection of *Paracoccidioides brasiliensis´* genome in biological samples by quantitative chain reaction polymerase (qPCR)

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ABSTRACT

Paracoccidioidomycosis (PCM) is a chronic mycosis caused by the saprobic and dimorphic species *Paracoccidioides brasiliensis* and *P. lutzii*. This disease is prevalent in Latin American countries. PCM appears as a relevant concern and challenge for the mycologists, since until now there is no a methodology suitable for an efficient and safe diagnosis and species identification. Thus, the present study aimed to validate a methodology for PCM´s diagnosis, using quantitative Polymerase Chain Reaction (qPCR) through target amplification of the gene encoding the recombinant protein Pb27, a common protein to the both species *Paracoccidioides brasiliensis* and *P. lutzii*. The experiments were performed *in vitro* to determine the specificity, efficiency and detection limit of qPCR assay, using specific primers and probe, which sequences were subject to a patent deposited in Brazilian CTIT, under the registration number: BR1020160078830. According to the results the technique showed sensitivity of 94% and specificity of 100%, demonstrating that it will be possible to develop a new fast and safe diagnostic PCM and can be standardized in order to present a low cost, accessible to the patient served by the public health system in Brazil and Latin America.

1. Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis, caused by the fungi *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*. These fungi are saprobic organisms that live in the soil absorbing nutrients from decomposing organic matter. Air is the vehicle of dispersion of its spores [1, 2]. Once inhaled, the spores will lodge in the lungs, which incubation period can range from one month to many years. The disease can be manifested by chronic pneumonia, with cough, nocturnal fevers and chronic hoarseness [3, 4]. However, the disease can be spread in disseminated form, affecting the liver, spleen, skin and mucous membranes [5-7].

The clinical signs of paracoccidioidomycosis can be confused with leishmaniasis, erythematous lupus, tuberculosis, syphilis, sarcoidosis, leprosy, in addition to other mycoses such as coccidioidomycosis, sporotrichosis and blastomycosis [8, 9]. Therefore, histopathological examination and culture of causative agent are important to clarify the diagnosis. It can be collected different biological samples, as skin biopsy, mucosa, ganglion aspirates, sputum and others. However, these tests are time-consuming and have low sensitivity [9, 10].

Due to the need to achieve a safe and rapid PCM´s diagnosis, several studies have been developed [11]. Serological tests, using the recombinant protein Pb27 as antigen specific to the *Paracoccidioides* complex, have presented satisfactory index of specificity and sensitivity [12, 26]. However, there are many cases in which serological tests cannot assist in diagnosiśs conclusion, due to the patient's immunosuppression profile [13]. So, in view of these difficulties and the growing number of people developing the disease, there is a need to develop molecular biology methodologies in order to detect genome of the agent in patient's biological samples. With this purpose, different genome targets have been studied, such as microsatellites [14]; regions of ribosomal DNA (rDNA), gene which expresses glycoprotein gp43 and other, demonstrating that PCR is a good tool to be used for the diagnosis and monitoring of treatment [15, 16].

In this study, we validated the quantitative PCR technique (qPCR) for molecular detection of *Paracoccidioides brasiliensis* in different biological samples. The qPCR was developed using the TaqMan® system with a species-specific probe for Pb27 protein gene [17].

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2. Materials and methods

2.1. Biological samples

Samples were collected from 147 patients seen at the Unified Health System of the State of Minas Gerais, Brazil. The study was approved by the Committee of Ethics in Research with human beings of the Santa Casa de Belo Horizonte Hospital, with the number: CAAE: 55549216.2.0000.5138. Among these samples, 78 had a confirmed diagnosis for PCM by clinical criteria, histopathological examination (50) and/or by serology (75), using the Radial Immunodiffusion technique. The Radial Immunodifusion was performed with GP43 provided by the Technological Center of the Federal University of Paraná. The clinical samples were distributed between 56 whole blood samples, 44 serum samples,12 biopsy samples, 3 puncture samples and 3 alveolar bronchial lavage (BAL) samples. The patient group consisted of 82% of males (64 male and 14 female).

The PCM negative-control group consisted of 69 patients with confirmed diagnosis for other diseases such as lymphoma, sarcoidosis, leishmaniasis, tuberculosis, bacterial infection, leprosy, lupus, other systemic mycoses and healthy patients. This group consisted of 30 women and 39 men.

2.2. DNA extraction from whole blood and biopsy

The DNA was extracted using the Ludwig kit (Brazil), with addition of beads to break the wall of fungís cells, as standardized by Graciele-Melo *et al*, 2016 [18]. For biopsy extraction it was necessary to treat the tissue with 10 ng of Proteinase K, overnight, prior to extracting DNA.

After extracted and purified, the DNA was assayed by spectrophotometry using Nanovue Plus (GE Healthcare Life Sciences, Sweden).

2.3. Quantitative PCR real time (qPCR)

For the real-time PCR reactions, was used the TaqMan® system (Applied Biosystems, USA), using primers targeted to Pb27 Pb255 gene region 3′- GCACACCATTCCCGCATACA-5 '; and Pb411 3′-TTTGACCTT CTGACCGTTGA-5′ probe PB27JA - 6FAM-TGACTACCAAAGCTGGCC GCA-BHQ1 [17].

The reaction was realized in 7500 fast equipment of Applied Biosystems (USA).The universal cycling condition was used for amplification (95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min). All samples were analyzed in duplicate. For all PCR reactions were used specific primers for the constitutive *β-globin* gene, as a quality control of the reaction. The negative and positive controls (*P. brasiliensis*- Pb18 and *P. lutzii*– Pb01) were included in all PCR reactions performed. The samples were considered positive allowing to the amplification end result, ever since the technique was standardized using specific probe, the negative samples did not amplify.

2.3.1. Sensitivity and specificity

Sensitivity was calculated by the ratio of positive results in qPCR among the PCM-positive samples. The specificity was calculated by the ratio between the negative qPCR results among the control PCM-negative samples. Accuracy was given by the sum of the positive and negative results divided by the total N of the samples [19, 20]. The indexes were calculated using the software IBM SPSS Statistic v20.

3. Results

Among the 56 blood samples analyzed, 51 (91%) were positive and 5 (9%) were negative; among the 44 serum samples, 40 (91%) were positive and 4 (9%) were negative; among the 12 biopsy samples, 3 puncture and 3 BAL samples, all of them (100%) were positive. All 69 samples from the PCM negative-control group were negative at qPCR (Fig. 1).

The mean Cq of the positive samples was 32.5, but the Cq distribution was heterogeneous (Fig. 2). According to the calculations, the sensitivity of the qPCR was 94%, the specificity was 100% and the accuracy (A) was 97%. The sensitivity confidence interval is 88%–100% with standard error of 0.03.

4. Discussion

This study was composed of a greater proportion of men (82%) in relation to women, corroborating with what is already described in other studies [21-23].

The qPCR validated in this study showed sensitivity of 94%, specificity 100% and accuracy 97%. To date, this test is among the published PCM´s diagnostic methods with the highest sensitivity and specificity. The IDRD, using the Gp43 antigen as a specific target, has sensitivity of 84% and specificity of 98% [24]. Western blotting, also using the gp43 antigen has a sensitivity of 93.6% and specificity of 80% [25]. However, it is not always possible to use purified antigen in the clinical routine, so in practice these values may suffer from oscillations. The Dot Blot test, using the Pb27 antigen, obtained sensitivity of 100% and specificity of 98% [26]. Kamikawa et al. (2017) standardized and validated the Dot ELISA using as antigen the yeast culture filtrate, showing specificity of 95.4%, sensitivity of 91% and accuracy of 93% [27]. Even with these good sensitivity indices, serological tests are not yet offered by commercial clinical laboratories, but are available to the population at the reference research centers and LACENS distributed in Brazil [28].

One of advantages of qPCR is that it can be performed with DNA extracted from any biological sample [11] and, in this way; the collection can be adapted to the clinical form of the disease. In this study, it is worth mentioning that among the 56 blood samples 51 (91%) are positive and 5 (9%) are negative; Among the 44 serum samples 40 (91%) were positive and 4 (9%) were negative; among the biopsy samples all 17 (100%) were positive.

Overall qPCR was positive in 74 (94.9%) samples and negative in 4 (5.1%). Among these four patients presenting negative results, one patient had been under treatment for 3 years and therefore, presented a negative result. Treatment lasts from months to years, depending on the decision of each referral center [29]. In many cases, the patient could be in follow-up, but without need, because he could already be cured. This fact leads us to believe that rt-PCR results could be used as cure criteria to follow the treatment. Currently, there is no well-established cure criterion for PCM [23]. So, we suggest that a study would be necessary to standardize these parameters using qPCR.

Regarding the other 3 negative samples, the patients presented the unifocal pneumonia and lymphocutaneous forms of the PCM. In these cases were not possible to detect the genome of the fungus in peripheral blood, since it is not the disseminated form of the disease. For these cases, qPCR can be performed with DNA extracted from the affected site, as biopsy, sputum or BAL. In one of the samples the blood qPCR result was negative and the biopsy was positive. This is due to the fact that when the fungus is restricted to an organ such as the lung or lymph nodes, there are no circulating cells in the blood stream.

The qPCR using the primers Pb255 and Pb411 and the probe PB27JA was 100% specific for detection of the fungus *Paracoccidioides brasiliensis* in biological samples. The clinical presentation of paracoccidioidomycosis can be confused with other autoimmune and infectious diseases [8, 9]. Among the 69 samples from the PCM negative-control group, there was no false positive. With this specificity value we can infer that this test is safe and can be used as a tool in differential diagnosis.

The qPCR has been increasingly standardized for diagnosis of systemic mycoses. Currently, this method represents an important tool because it can detect very low amounts of circulating DNA of the causal agent in a clinical sample [17, 30, 31].

An important step in validating a diagnostic test is the cut-off

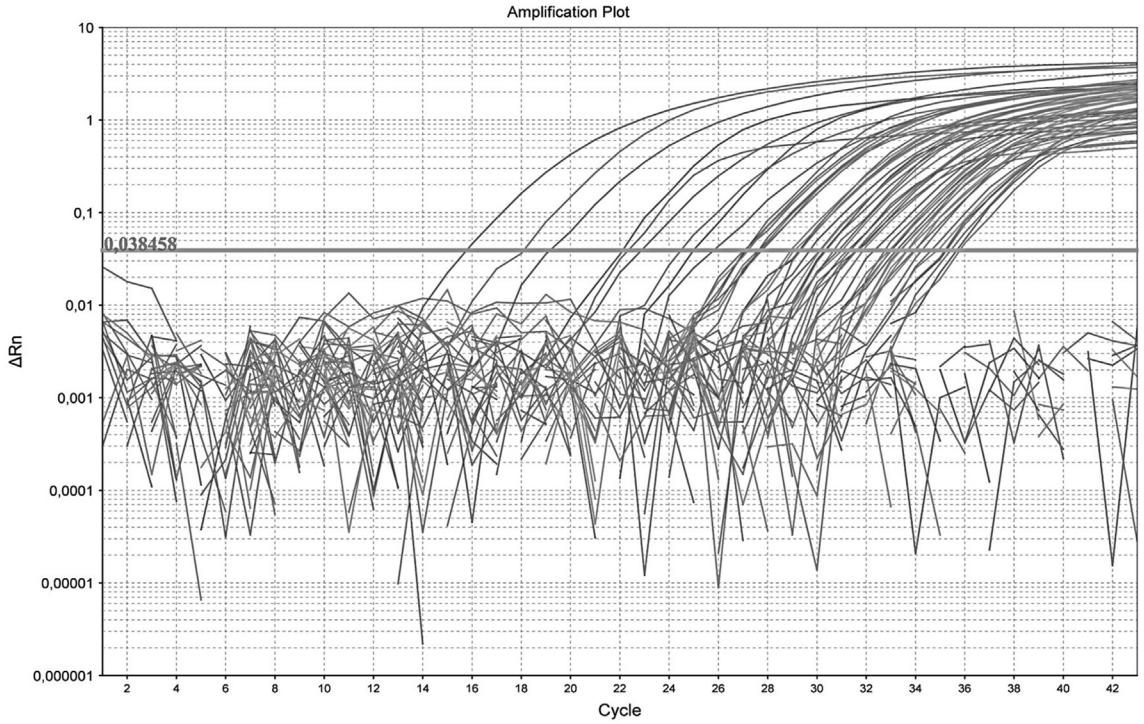


Fig. 1. Amplification Plot Above the threshold (the horizontal line - ΔRn 0,038458), are the positive samples and positive controls; below the threshold, are the negative samples and negative controls, without amplification.

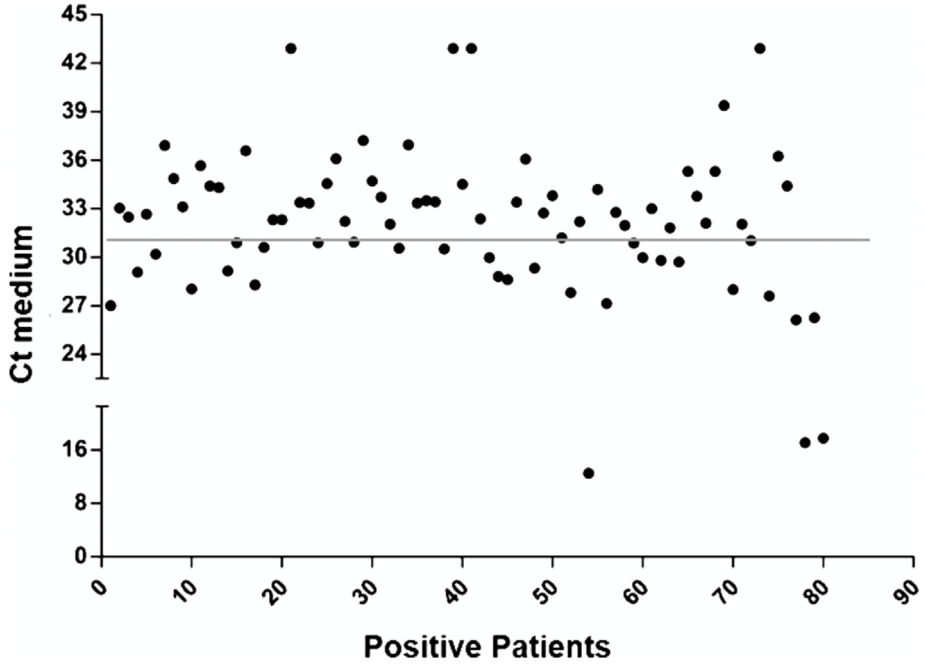


Fig. 2. Cq distribution of positive samples SPSS software.
Cq mean amplification of the positive samples, threshold line, Cq32,5.

determination. However, in qPCR using the probes system the negative does not amplify, because there is no complementarity of the probe and the primers, which decreases the possibility of formation of non-specific products [11]. However, we observed that the standard curve points that amplified from Cq 35 were not consistent in the relationship between DNA mass and Cq. This data corroborates with TOO (2003), which does not advise considering values of Cqs higher than 35 [32]. But it is worth mentioning that Cq values can vary according to the amount of DNA mass of the fungus present in the sample. Therefore it is worth taking into account the clinic and / or fact that the patient is or is not under treatment. Patients undergoing treatment and / or with few circulating fungal cells consequently presented higher Cq values, which may be considered if it complements other diagnostic parameters, supporting in the therapeutic decision of the physician. It is worth noting that qPCR is a test that only assists in the diagnosis and, therefore, needs to be associated with other tests to close the diagnosis, such as direct examination and fungus culture.

The results of the standardization of qPCR show high sensibility

(94%), high specificity between the diseases compared (100%), demonstrating that this test is promisor for use in the axillar diagnosis for PCM. The qPCR of whole blood DNA is very efficient to detect whether the infection is disseminated or not, directing better the treatment and the care with the patient. In the guideline for PCM are well described methodologies for PCM´s diagnosis [22]. Afterward, we could consider that in the next future, qPCR could be included for support PCM´s diagnosis and be widely used in health services, since it can clarify the diagnosis of PCM, especially in cases where clinical, serological and mycological tests cannot elucidate.

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Conflicts of interest

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2018.05.039.

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