

An optimized one-tube, semi-nested PCR assay for *Paracoccidioides brasiliensis* detection

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ABSTRACT

Introduction: Herein, we report a one-tube, semi-nested-polymerase chain reaction (OTsn-PCR) assay for the detection of *Paracoccidioides brasiliensis*. **Methods:** We developed the OTsn-PCR assay for the detection of *P. brasiliensis* in clinical specimens and compared it with other PCR methods. **Results:** The OTsn-PCR assay was positive for all clinical samples, and the detection limit was better or equivalent to the other nested or semi-nested PCR methods for *P. brasiliensis* detection. **Conclusions:** The OTsn-PCR assay described in this paper has a detection limit similar to other reactions for the molecular detection of *P. brasiliensis*, but this approach is faster and less prone to contamination than other conventional nested or semi-nested PCR assays.

Keywords: Mycosis. Molecular diagnosis. Polymerase chain reaction assays.

Paracoccidioidomycosis (PCM) is an endemic infection in Latin America and is caused by *Paracoccidioides brasiliensis*, a thermal-dimorphic fungus¹. The distribution of infections is restricted to Central and South America and is observed between Mexico (23°N) and Argentina (34°S)¹. Brazil is the country with the largest number of cases of the disease, with an estimated incidence ranging between 10-30 cases/million inhabitants and a mortality rate of 1.4 deaths/million inhabitants². PCM diagnosis is based on clinical symptoms, direct microscopic examination of clinical specimens, isolation of fungus in culture and the detection of specific antibodies by serological techniques³. However, these methods are dependent on the skills of laboratory staff, the capacity for the organism to be cultured and the cross-reactivity with antigens of other fungi³. Thus, several new molecular methods have been developed for the detection of *P. brasiliensis*, including polymerase chain reaction (PCR) assays^{3,4}.

In this study, we have proposed a one-tube, semi-nested PCR (OTsn-PCR) approach for the detection of *P. brasiliensis*, which is as efficient as classical nested and semi-nested PCR assays and can be adapted for the detection of other pathogenic fungi.

For this study, we used the following microorganisms: *P. brasiliensis* (LDR 1 and Pb 18 strains), *Cryptococcus* sp., *Sporothrix* spp., *Histoplasma capsulatum*, *Candida albicans*

(strain CR15) and *Trichophyton rubrum*. In addition, positive sputum samples from 14 patients with Paracoccidioidomycosis, diagnosed by direct observation of fungal cells and clinical signs (including a productive cough and weight loss), were obtained from the University Hospital of Western Paraná State University (UNIOESTE, Paraná, Brazil). This study was approved by the Western Paraná State University Ethics Committee for Human Beings (UNIOESTE - N° 27373/2009).

Deoxyribonucleic acid (DNA) from yeast-phase fungal cells and clinical samples were extracted by cell lysis via a liquid nitrogen freeze-thaw, followed by phenol-chloroform treatment and sodium acetate-ethanol precipitation as described by Koishi et al.⁵ The DNA concentration and purity were determined by spectrophotometry measurement at 260/280nm. Due to the possible presence of inhibitors, the quality of DNA extracted from the clinical samples was evaluated by PCR using two primers specific to the human CXCR4 gene; SDF1 (5'-CAGTCAACCTGGGCAAAGCC-3') and SDF2 (5'-CCTGAGAGTCCTTTTGC GGG-3')⁶.

The OTsn-PCR approach was developed to detect the internal transcribed spacer regions (ITS) of fungi. The first step was performed with primers termed ITS13B (5'-GTTTCCGTAGGTGAACCTGCGG-3'), a modified form of an ITS1 primer, and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), as described by White et al.⁷ The second step of the OTsn-PCR assay was performed using the ITS13B primer and a species-specific primer. We tested a species-specific primer for *P. brasiliensis*, termed MJ034B (5'-ATAGGTCTCAGACGTCAAAGCTCC-3'), a modified form of the MJ03 primer described by Koishi et al.⁵, and a species-specific primer for *H. capsulatum*, which we termed HC013B (5'-TCATGCTCAGACGCCAATCGTTC-3').

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The reaction was performed with 1X Buffer (20mM Tris-HCl [pH 8.4] and 50mM KCl), 2mM of each deoxynucleotide triphosphate (dNTP), 2mM MgCl₂, 1μM ITS13B primer and a species-specific primer, 0.1μM ITS4 primer, 0.1mg/ml of purified gelatin, 1U Taq DNA polymerase and ultrapure water to a final volume of 25μl. DNA from *Cryptococcus* sp., *Sporothrix* spp., *C. albicans* (strain CR15) and *T. rubrum* and ultrapure water were used as negative controls. Additionally, 2.5ng of purified DNA from *P. brasiliensis* or *H. capsulatum* were used as positive controls. The PCR conditions were as follows: 95°C for 2min (for the initial denaturation of the DNA); 35 cycles of 95°C for 30sec, 55°C for 30sec and 72°C for 1min (initial amplification phase); 35 cycles of 95°C for 30sec, 72°C for 30sec, and 72°C for 1min (secondary amplification phase); and a final extension at 72°C for 5min.

This OTsn-PCR approach was compared with the nested and semi-nested PCR methods described by Imai et al.⁸, Theodoro et al.⁹ and Koishi et al.⁵ Similar to our OTsn-PCR assay, these reactions use sequences of ribosomal DNA as targets, especially from the ITS, and are specific for the detection of *P. brasiliensis*. For the sake of comparison, we used five different serially diluted *P. brasiliensis* DNA concentrations (2.5ng, 250pg, 25pg, 2.5pg and 0.25pg). All of the protocols were repeated at least three times, as originally described. Furthermore, to compare cycle number effects on the limit of detection among the methods, the methods developed by Imai et al.⁸ and Theodoro et al.⁹ were also performed using 35 cycles.

Considering the high incidence of fungal infections and the expanding spectrum of fungal pathogens, it is essential to develop methods for the early and accurate identification of the causative agents of fungal infections. Recently, several semi-nested and nested PCR assays have been developed for fungal detection¹⁰. However, these reactions are time consuming and susceptible to accidental contamination during the transfer of aliquots from the first reaction mixture to new tubes used for the second reaction¹¹⁻¹³.

Using a serial dilution of purified DNA from *P. brasiliensis* (from 2.5ng to 0.25pg), the OTsn-PCR assay described in this study was able to detect up to 2.5pg of *P. brasiliensis* DNA (Figure 1A) without cross-reactivity with other fungi (data not shown). The limit of detection for the OTsn-PCR assay was comparable with that of the protocol developed by Koishi et al.⁵ (Figure 1B) and was more sensitive than the protocols in

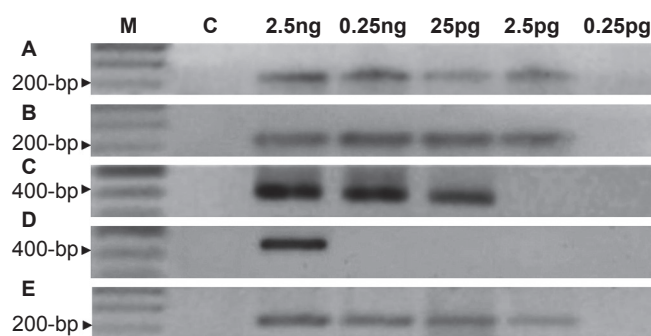


FIGURE 1 - Sensitivity of the OTsn-PCR assay for identifying *Paracoccidioides brasiliensis* (A) when compared with the nested or semi-nested PCR reactions developed by Koishi et al. (B), Theodoro et al. (C) and Imai et al. (D). Sensitivity of the OTsn-PCR assay for the identification of *Histoplasma capsulatum* (E). OTsn-PCR: one-tube semi-nested-polymerase chain reaction; M: 100-bp DNA Ladder (Invitrogen, Brazil); C: negative control. The agarose gel was stained with SYBR SAFE (Invitrogen, Brazil).

the Imai et al.⁸ and Theodoro et al.⁹ studies (Figures 1C-1D). Furthermore, when using DNA extracted from the clinical samples, the OTsn-PCR assay and the protocol developed by Koishi et al.⁵ were able to detect *P. brasiliensis* DNA in all of the samples (100%) (Table 1), while the protocols of Imai et al.⁸ and Theodoro et al.⁹ only detected *P. brasiliensis* DNA in four (28.5%) and eight (57.2%) of the clinical samples, respectively (Table 1). A possible cause for this difference may be the lower numbers of cycles used in these reactions. For this reason, all of samples were retested following the Imai et al.⁸ and Theodoro et al.⁹ PCR methods using 35 cycles. The numbers of positive samples increased from four to six (42.8%) and from eight to 10 (71.4%) using the PCR methods of Imai et al.⁸ and Theodoro et al.⁹, respectively (Table 1). However, the methods developed by Imai et al.⁸ and Theodoro et al.⁹ failed to detect *P. brasiliensis* DNA in some of the *P. brasiliensis*-positive clinical specimens.

In general, nested or semi-nested PCR assays performed in a single tube are less sensitive when compared with assays that take place in two stages and in separate tubes¹¹⁻¹³. However, in our study, we achieved the same limit of detection for the OTsn-PCR assay as the semi-nested PCR approach described by Koishi et al.⁵, which was only possible due to the use of gelatin in the reaction. When the OTsn-PCR assay was performed

TABLE 1 - Detection of *Paracoccidioides brasiliensis* in clinical (sputum) samples from 14 patients. Comparison of the OTsn-PCR assay with the nested or semi-nested PCR assays developed by Koishi et al.⁵, Theodoro et al.⁹ and Imai et al.⁸.

Protocols	Positive		Negative	
	n	%	n	%
Imai et al.	4 (6)*	28.5 (42.8)	10 (8)	71.5 (57.2)
Theodoro et al.	8 (10)	57.2 (71.4)	6 (4)	42.8 (28.6)
Koishi et al.	14	100.0	0	0
OTsn-PCR	14	100.0	0	0

OTsn-PCR: one-tube semi-nested-polymerase chain reaction. *Methods of Imai et al. and Theodoro et al. using 35 cycles.

without gelatin, the limit of DNA detection was 0.25ng (data not shown). Despite the use of gelatin in some studies, its role in these types of PCR reactions remains unknown¹¹.

To assess the use of the OTsn-PCR assay for the detection of other fungi, we modified the assay to specifically detect *H. capsulatum* DNA by changing only the species-specific primer used in the reaction. The detection limit of this assay was as low as 2.5pg (**Figure 1E**), which was similar to that observed when the *P. brasiliensis* species-specific primer was used. No cross-reactivity with the DNA of other fungi was observed.

In conclusion, our results demonstrate that the OTsn-PCR assay described in this study has a limit of detection identical to or better than the conventional nested and semi-nested PCR assays for *P. brasiliensis* detection and can easily be adapted for the detection of any pathogenic fungi simply by changing the species-specific primer. This approach will be a focus in our future studies. Furthermore, this reaction is faster and less prone to contamination than the conventional nested or semi-nested PCR assays. Thus, the OTsn-PCR assay has great potential for laboratory PCM diagnoses.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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