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Detection of *Paracoccidioides brasiliensis* gp43 Gene in Sputa by Loop-Mediated Isothermal Amplification Method

B. T. Tatibana,[1] A. Sano,[2] J. Uno,[2] K. Kamei,[2] T. Igarashi,[2] Y. Mikami,[2] M. Miyaji,[2] K. Nishimura,[2] and E. N. Itano[3]*

[1]*Microbiology Post Graduation Program, State University of Londrina, Londrina, PR, Brazil*
[2]*Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan*
[3]*Department of Pathology Sciences, CCB, State University of Londrina - Londrina, PR, Brazil*

The fungus *Paracoccidioides brasiliensis* is the pathogen of paracoccidioidomycosis (PCM), a systemic mycosis prevalent in Latin America. The loop-mediated isothermal amplification method (LAMP) was used in this study to detect the presence of *P. brasiliensis* in sputa samples from patients with chronic PCM, suspected PCM, and a negative control. The target *P. brasiliensis* gp43 gene was amplified in less than 4 hr in 11 of 18 sputa samples tested. The LAMP method had the advantage of speed and simplicity compared with the classic diagnostic methods such as the histopathological test or biological material culture and did not require sophisticated technical apparatus. It would be an important aid in cases where immediate treatment would mean patient survival, especially in immune-suppressed patients. J. Clin. Lab. Anal. 23 : 139–143, 2009. © 2009 Wiley-Liss, Inc.

Key words: gp43 gene; *Paracoccidioides brasiliensis*; paracoccidiomycosis; LAMP

INTRODUCTION

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the *Paracoccidiodes brasiliensis* (*P. brasiliensis*) fungus. To date, the natural habitat and saprophytic life conditions of *P. brasiliensis* have not been totally clarified (1). There are reports of isolation from penguin feces from Antarctica (2), soil samples (3), dog meal, soil-contaminated (4), fructivore bat gut (5), and armadillo gut (6).

The geographic distribution of the mycosis is restricted to Latin American countries (5,7). As it is not a disease with obligatory notification, its true prevalence cannot be calculated. In endemic countries, such as Brazil, it is estimated that the annual rate of incidence in the population is 1–3 per 100,000 inhabitants and mortality of 0.14 per 100,000 inhabitants (8). It is estimated that in these regions there are approximately 10 million persons infected by *P. brasiliensis* and most have no clinical symptoms (1).

The PCM infection mechanism occurs by conidia installation, which can be destroyed in the lung parenchyma or produce a focus of infection drained to the lymph node region, forming the primary infection complex. It can disseminate by the hematogenic and/or lymphatic path, reaching other organs and causing the juvenile or acute form (9) or remain with a primary scar focus with viable fungi called a "quiescent lesion," which evolves to chronic PCM (10).

Diagnosis is based on culture, histopathology, and antibody detection in clinical samples. The latter can be problematic depending on the antigen used, with the occurrence of cross-reaction, and false-positive or false-negative results (11). In cases of patients with immune problems, although it is not considered an opportunist fungus, immunological diagnosis can be hindered by reduced antibody production. In histological sections, *P. brasiliensis* might be missed or confused with other dimorphic fungi such as *Histoplasma* spp. or *Coccidioides immitis* (12).

In non-endemic areas, diagnosis is problematic for clinicians, pathologists, and microbiologists unfamiliar

*Correspondence to: E. N. Itano, Departamento de Ciências Patológicas, CCB, Universidade Estadual de Londrina, Campus Universitário, 86051-970 Londrina, PR, Brazil. E-mail: itanoeiko@hotmail.com

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with PCM and its etiological agent (13). To improve the sensitivity and specificity of PCM diagnosis, *P. brasiliensis* identification has been attempted by molecular techniques. Recently the LAMP technique was used by Endo et al. (14) to identify the species-specific gene gp43 of *P. brasiliensis* without cross-reaction with other fungus isolates. Using only one sample of histological cuts, it was suggested that the method could be used in other types of clinical samples.

The LAMP method was assessed in this study for PCM diagnosis in human sputa samples using the same primers that were used by Endo et al. (14). It is a fast and specific alternative method; the use of sputa is a non-invasive sample collection technique and does not expose the patients to risks. It can be used as a complementary technique especially in cases where a delay in diagnosis may put patients at risk.

MATERIALS AND METHODS

Clinical Sputa Specimens

The sputa samples obtained from 17 patients with chronic PCM (male, aged 28–72 years) with a diagnosis established by X-ray, clinical data, and isolation by culture and/or serological tests (Table 1, samples 2–16 and 18–19), one suspected PCM (Table 1, sample 17) and 01 negative PCM control (Table 1, sample 20) from the Pneumology and Infectious Disease Sector, University Hospital, State University of Londrina, Londrina, Paraná, and Sanitary Pneumology Sector of Integral Infection-contagious Center, Municipal Health Service, Londrina, Paraná were collected in Brazil between November 2000 and June 2003. The backgrounds are shown in Table 1 (Samples 2–20). The sputa were defrosted and fixed with 70% ethanol overnight, dried in Brazil at room temperature, and analyzed in Japan. The dried sputa were washed with distilled water three times with centrifuging, and processed for DNA extraction.

A 44–year-old male who was 3rd generation of Japanese-descendant Brazilian was diagnosed with PCM by clinical manifestation, cytological observation of the sputum, and fungal culture in Japan (15). The preliminary study for detection of gp43 of *P. brasiliensis* from the sputum by the LAMP method was positive (Table 1, sample 1). The sputum was fixed with 70% ethanol for overnight, rinsed with distilled water three times, and processed for DNA extraction.

Extraction of DNA From Sputa

DNA was extracted with DEXPAT® (TaKaRa Biomedical, Inc., Ohtsu, Japan) according to the manufacturer's instructions. Five hundred microliters of the extract solution was added to the washed pellet of sputum, whose volume was approximately 50 µl. The mixture was incubated at 100°C for 10 min and

TABLE 1. Patient's backgrounds and results of diagnoses

				Immunological test				
Sample number	Age	Involvement	Chest X-ray	ELISA	ID	Cytology or biopsy	Fungal isolation	LAMP
1	44	P	+	ND	ND	+	+	+
2	65	M	+	1/800	1/16	+	+	–
3	46	M	+	1/400	1/4	+	–	–
4	45	MP	+	1/200	1	+	–	+
5	50	M	+	1/400	1/8	+	–	+
6	72	P	+	1/800	1/16	–	–	–
7	53	P	+	1/400	–	–	–	+
8	65	L	+	1/200	1/8	–	–	+
9	46	M	+	1/200	–	+	–	+
10	67	M	+	1/800	1/8	–	–	+
11	70	P	+	1/800	–	+	–	+
12	52	P	+	1/800	1/16	+	–	–
13	51	M	+	1/800	1/4	+	–	–
14	41	P	+	1/200	1/4	–	–	+
15	50	M	+	1/400	–	–	–	+
16	50	M	+	1/800	1	+	+	–
17	70	P	+	1/800	–	–	–	–
18	40	P	+	1/200	–	–	–	+
19	77	P	+	1/800	1	–	–	–
20	28	Control	–	–	–	ND	ND	–

All patients are male. P = pulmonary involvement, M = mucocutaneous involvement, and L = lynphatic involvements (ND = not done; – = negative result). 1 = Patient diagnosed in Japan, 2–19 = patient diagnosed in Brazil, and 20 = healthy control.

centrifuged at 12,000 rpm (13,201 *g*) for 10 min. The supernatant was then processed for the LAMP.

LAMP Method

The LAMP method used in this study was developed by Endo et al. (14) and detects the gp43 gene with a combination of F3 for LAMP, B3, FIP, and BIP primers designed from the partial sequence of gp43 (GenBank accession number U26160) by a registration system primer designing web site (FUJITSU Ltd., Tokyo, Japan: "LAMP PIMER EXPLORER" web site in "Netlaboratory" homepage http://venus.netlaboratory.com/partner/lamp/index.html). These primers recognize an area of gp43 where variation among strains has not been reported.

The primer sequences were as follows: F3 for LAMP, 5-TCA CGT CGC ATC TCA CAT TG-3 used in the species-specific forward primer; B3, 5-AAG CGC CTT GTC CAA ATA GTC GA-3 used in the species-specific reverse one; FIP, 5′-TGG CTC CAG CAA TAG CCA CCC GTC AAG CAG GAT CAG CAA T-3′ designed from the forward sequence of 425th–445th and the complementary sequence of 464th–485th; and BIP: 5′-CAT GTC AGG ATC CCG ATC GGG CCT TGT ACA TAT GGC TCT CCC T-3′ designed from the forward sequence from 648th to 668th and the complementary sequence from 691st to 712th. One microliter of 10 ng/ml DNA template, and 40 pmol each of the FIP and BIP primers, and 5 pmol each of the F3 for LAMP and B3 primers were mixed with 12.5 µl of 2 reaction mix in the kit (Loop AMP, Eiken Chemical Co., Ltd., Tokyo, Japan) in a final volume of 23.0 µl. DNA mixtures were incubated at 63°C for 120 min. The reaction was stopped by heating the mixture at 80°C for 2 min to inactivate the enzyme of LAMP amplification. The amplified products were electrophoresed in 1.0% agarose gels in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA) and stained with ethidium bromide. DNA extracted from a *P. brasiliensis* isolate (IFM 41621, Pb-18) was used as a positive control for the amplification.

Double Immunodiffusion in Gel

Six glass slides (2.5 × 7.5 cm) were covered with a 3.0 mm-thick layer of agar (1% agar, 0.85% NaCl, and 0.02% sodium azide) with seven wells (one central and six peripheral). The *P. brasiliensis* exoantigen sample was applied to the central well, and serum sample in duplicate with no dilution, and 1:2–1:32 dilutions were applied to the peripheral wells. The samples were incubated in a humid chamber at room temperature for 24 hr. The gel was washed, dried, and stained with amido black.

RESULTS

LAMP-amplified products appeared as a ladder of bands on electrophoretic gels. The preliminary trial of the sputum from a Japanese patient is shown in Figure 1(a). It was possible to amplify the target gene in 11 out of 18 sputa samples from Brazilian PCM (Fig. 1: (b, c)).

LAMP-positive samples included cases that were negative by cytology/biopsy and fungal isolation (Table 1). Without correlation to LAMP, 12 out of 18 patients were positive in immunodiffusion (ID) test for PCM. LAMP-negative samples included 8 samples that are positive by ELISA (Table 1 samples 2, 3, 6, 12, 13, 16, and 19), 7 samples positive by ID (Table 1 samples 2, 3, 6, 12, 13, 16, and 19), by biopsy (Table 1 samples 2, 3, 12, 13, and 16) and by biological material culture (Table 1 samples 2 and 16).

DISCUSSION

There is difference in sensitivity and specificity for immunological test to confirm PCM diagnosis. Morphological and physiological characteristics used in classic diagnostic methods are complex. Developing simpler and complementary techniques has become important, especially due to the increase in the number of immune-suppressed patients. Therefore, a clear diagnosis of fungal infections is necessary.

The certainty diagnosis of PCM consists in showing the pathogen in histological preparations, visualization in fresh or in culture exams (16). However, there are cases where physical or clinical state prevents access to the lesion because invasive methods are usually used for collection. In these cases, serological techniques provide important auxiliary diagnosis. Although the serological techniques such as ID and ELISA are used to confirm PCM diagnosis, the rates of false-positive and false-negative results are still very high, and the specificity and sensitivity of the technique are directly related to the antigen or antibody used (17).

ID was the method used by clinical laboratories because it is an easy procedure, but the results can vary because of different parameters, including the antigen preparation, the form of the disease, and the starting of treatment (18). The follow-up of patients receiving treatment for PCM has shown that the antibody titers obtained by the ID test frequently do not correlate with the clinical status of the patient (19). In some patients, high antibody titers were observed until the end of the treatment when the patients were clinically cured. On the other hand, low antibody titers are related to the absence of clinical symptoms in most patients. However, in some cases low titers are present although clinical symptoms are present (20).

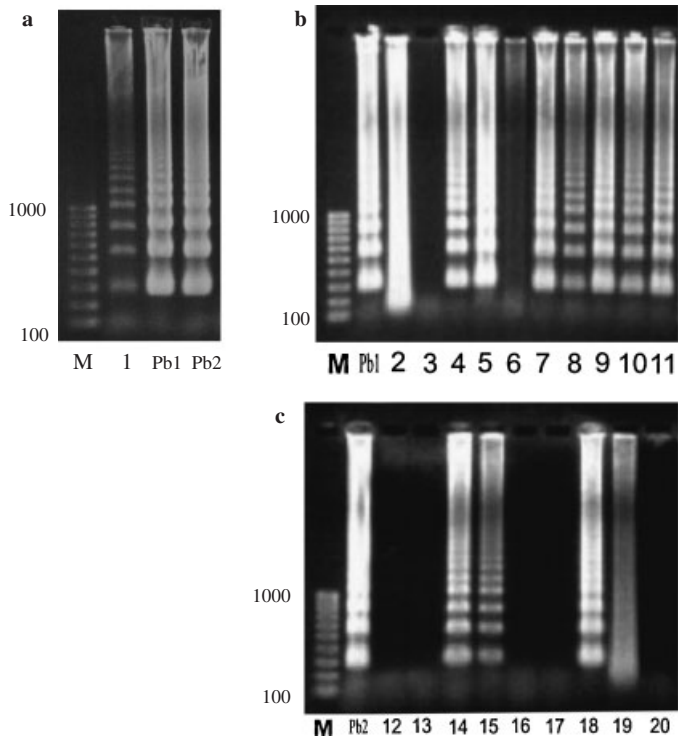

Fig. 1. The detection of gp43 gene of *P. brasiliensis* by the LAMP method. **(a)** 1 = First PCM case diagnosed in Japan by LAMP; **(b, c)** 2–19 = Brazilian PCM patients and 20 = Healthy control (Table 1). The specific DNA banding patterns for the gp43 gene of *P. brasiliensis*: Pb1 = IFM 41621, Pb2 = Pb 18, and M = marker.

The ELISA test has been used to detect antibodies in most systemic mycosis. In spite of this, compared with the immune diagnosis of PCM, the technique still offers high percentages of crossed reactivity, especially in patients with histoplasmosis, candidiasis, "Jorge Lobo" disease, and recently against sera from apparently healthy persons, resident in PCM endemic areas (11).

Against other molecular tests, LAMP has shown the advantage of greater sensitivity, because using clinical samples and the same target *P. brasiliensis* DNA, a minimum of 1 ng was detected by the PCR technique (21), whereas in LAMP, the minimum sensitivity reported was 100 fg (14). Moreover, LAMP does not require sophisticated technical apparatus, neither high biosafety.

In the first instance, the results presented here can be interpreted as false negatives or with low sensitivity because from 18 potentially positive samples, 11 were confirmed. However, when analyzed case-by-case, we can state that the LAMP method was successful in the proposed task.

The negative sample by LAMP number 17 (Table 1) was from a patient who presented a non-specific lung clinical condition, later diagnosed as candidiasis, which explained the ELISA reagent by a crossed reaction. This assertion was supported by the non-demonstration of *P. brasiliensis* on the histopathological tests and negative attempt of isolation by culture.

Most samples (Table 1 samples 2, 3, 6, 13, and 16), before collecting the sputa samples, received medication treatment with sulfametoxazol and trimetoprima, which explained the non-presence of the fungus in sputa but in the tissues confirmed by the highly reactive ELISA (majority with 1/800, except for sample 3) and the positive isolation in two biopsy tissue samples (Table 1, samples 2 and 16).

In group of negative LAMP, the form classified as lung (Table 1, sample 6) was negative for culture and for visualization of the fungus by histopathology, which was in fact, a sample from a patient considered cured. Two cases were of tuberculosis, where there was no *P. brasiliensis* isolation (Table 1 samples 12 and 19), only visualizing on a histopathology slide (Table 1 sample 12). This would be expected because the lesions can remain surrounded by tissue for years in lesions with viable fungi, which does not mean active fungemia (17). There is a possibility that, at the moment when the sputa samples were collected from this group, except on sample number 3 where the etiological agent was different, there was a PCM infection. The positive radiological result in these cases are due to so-called "radiological lung scars" (12).

On the other hand, the 11 positive samples were of PCM disease, in its chronic form, with lesions in the oropharinge, in the process of acute clinical condition. All these patients were from centers of reference for long-term PCM treatment, with an average of 10 years treatment, with previous diagnosis demonstration by biopsy or culture. Medication treatment was quickly established, which led to clinical improvement and normalization of serological tests (data not shown). Through assertive treatment, the results by culture were all negative. This supports the need for other techniques for *P. brasiliensis* detection, because although it is considered the diagnostic gold standard, the small trace of fungus found in the biopsy tissue can lead to non-isolation (9, 11, 17).

Thus, the LAMP method can complement PCM diagnosis. It has the advantage that cross-reactivity does not occur. It is fast, sensitive, and false-positive results do not occur because it uses a single diagnostic parameter. It is the detection of the single nucleotide sequence for *P. brasiliensis*, unlike some immunological tests that use indirect detection methodology of micro-organisms, which depends on many unknown factors of parasite–host relationship for certain diagnosis.

REFERENCES

1. Restrepo A, McEwen JG, Castaneda E. The habitat of *Paracoccidioides brasiliensis*: How far from solving the riddle? Med Mycol 2001;39:233–241.
2. Guezelle E. Aislamiento de *Paracoccidioides* sp. de heces de pinguino de la Antártida, res. B2. In: Resúmenes IV Encuentro Internacional sobre Paracoccidioidomicosis. Caracas: Venezuela, 1989.
3. Terçarioli GR, Bagagli E, Reis GM, et al. Ecological study of *Paracoccidioides brasiliensis* in soil: Growth ability, conidia production and molecular detection. BMC Microbiol 2007;7:92.
4. Ferreira MS, Freitas LH, Lacaz CS, et al. Isolation and characterization of a *Paracoccidioides brasiliensis* strain from a dog food probably contaminated with soil in Uberlandia, Brazil. J Med Vet Mycol 1990;28:253–256.
5. Grosse E, Tamsitt J. *Paracoccidioides brasiliensis* recovered from the intestinal tract of three bats (*Artibeus lituratus*) in Colombia S.A. Sabouraudia 1965;4:124–125.
6. Bagagli E, Sano A, Coelho KIR, et al. Isolation of *Paracoccidioides brasiliensis* from armadillos (*Dasypus novemcinctus*) in an endemic area of paracoccidioidomycosis. Am J Trop Med Hyg 1998;58:505–512.
7. Calle D, Rosero DS, Orozco LC, Camargo D, Castaneda E, Restrepo A. Paracoccidioidomycosis in Colombia: An ecological study. Epidemiol Infect 2001;126:309–315.
8. Coutinho ZF, Silva D, Lazera M, Petri V, Oliveira RM, Sabroza PCl. Paracoccidioidomycosis mortality in Brazil (1980–1995). Cad Saúde Pública 2002;18:1441–1454.
9. Londero AT, Del Negro G. Paracoccidioidomicose. J Pneumol 1986;12:41–60.
10. Franco M. Host-parasite relationships in paracoccidioidomycosis. J Med Vet Mycol 1986;25:5–18.
11. Elias Costa MR, Da Silva Lacaz C, Kawasaki M, De Camargo ZP. Conventional versus molecular diagnostic tests. Med Mycol 2000;38:139–145.
12. Wanke B, Londero AT. *Paracoccidioides brasiliensis*. In: L Ajello, RJ Hay, editors. Topley & Wilson's Microbiology and Microbial Infections, Medical Mycology, Vol. 4, Ninth edition. London, England: Arnold, 1998.
13. Ajello L, Polonelli L. Imported paracoccidioidomycosis: A public health problem in non-endemic areas. Eur J Epidemiol 1985;1:160–165.
14. Endo S, Komori T, Ricci G, et al. Detection of gp43 of *Paracoccidioides brasiliensis* by the loop-mediated isothermal amplification (LAMP) method. FEMS Microb Lett 2004;23: 93–97.
15. Igarashi T, Kurose T, Itabashi K, et al. A case of chronic pulmonary paracoccidioidomycosis. Nihon Kokyuki Gakkai Zasshi 2004;42:629–633.
16. Camargo ZP, Franco MF. Current knowledge on pathogenesis and immunodiagnosis of paracoccidioidomycosis. Rev Iberam Micol 2000;17:41–48.
17. Brummer E, Castaneda E, Restrepo A. Paracoccidioidomycosis: An update. Clin Microbiol Rev 1993;6:89–117.
18. Siqueira AM. Avaliação da sensibilidade e especificidade de algumas provas sorológicas no diagnóstico, prognóstico e controle de cura da paracoccidioidomicose. Caracterização do antígeno E_2 de *Paracoccidioides brasiliensis*. [Tese de Doutorado]. São Paulo: Universidade de São Paulo, 1982.
19. Neves AR, Mamoni RL, Rossi CL, Camargo ZP, Blotta MHS. Negative immunodiffusion test results obtained with sera of paracoccidioidomycosis patients may be related to low-affinity immunoglobulin G2 antibodies directed against carbohydrate epitopes. Clin Diagn Lab Immunol 2003;10:802–807.
20. Silva SHM, de Mattos Grosso D, Lopes JD, et al. Detection of *Paracoccidioides brasiliensis* gp70 circulating antigen and follow-up of patients undergoing antimycotic therapy. J Clin Microbiol 2004;42:4480–4486.
21. Gomes GM. Padronização da técnica de PCR para diagnostico de paracoccidioidomicose baseada em sequências do gene da gp43 de *Paracoccidioides brasiliensis*. [Dissertação de mestrado] São Paulo: Escola Paulista de Medicina, 1999.