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Detection of *gp43* of *Paracoccidioides brasiliensis* by the loop-mediated isothermal amplification (LAMP) method

Shigeo Endo [a], Takashi Komori [a,b], Giannina Ricci [c], Ayako Sano [a,*], Koji Yokoyama [a], Akira Ohori [a], Katsuhiko Kamei [a], Marcello Franco [c], Makoto Miyaji [a], Kazuko Nishimura [a]

[a] *Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8673, Japan*
[b] *Analytical Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan*
[c] *Departamento de Anatomia e Patologia, Faculdade de Medicina, Universidade Federal São Paulo, São Paulo, Brazil*

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Abstract

Paracoccidioidomycosis is a deep mycosis caused by the thermo-dependent dimorphic fungus *Paracoccidioides brasiliensis* and is prevalent in Latin American countries. We detected the species specific *gp43* gene of *P. brasiliensis* by loop-mediated isothermal amplification (LAMP) in 22 clinical and seven armadillo-derived isolates. The amplified DNA appeared as a ladder with a specific banding pattern. The advantage of the LAMP method is speed; only 3 h were necessary for identification of the organism and diagnosis of the disease. We were also able to obtain positive results from DNA extracted from a paraffin-embedded tissue sample of paracoccidioidomycosis, suggesting that this method may achieve clinical application in the near future.

Keywords: *gp43* gene; *Paracoccidioides brasiliensis*; paracoccidioidomycosis

1. Introduction

Paracoccidioidomycosis (PCM) is caused by the thermo-dependent dimorphic fungus, *Paracoccidioides brasiliensis*, which is endemic to Latin American countries and invades the skin, lymph nodes, visceral organs, such as the lung, and central nervous system [1]. PCM is uncommon, and diagnosis outside the endemic areas is often difficult. Most occurrences of PCM in Japan, for example were thought to be malignant tumors at the time of presentation, and the patients underwent biopsy [2]. A rapid and accurate method for diagnosis of PCM has been anticipated, and polymerase chain reaction (PCR) methods for detecting *P. brasiliensis* in blood, tissue, and histopathology samples have been reported recently. These PCR-based assays include amplification of the actin gene [3], 28S ribosomal RNA-specific gene [4], 5.8S ribosomal RNA (rRNA) gene including the internal transcribed spacers (ITS) 1 and 2 [5,6], and the *gp43* and *gp27* genes [5–9]. However, traditional PCR requires at least 5 or 6 h to detect specific genes in fungal cells, and more than 12 h may be required for nested PCR testing of blood or tissue samples. In the present study, we used a new method, called loop-mediated isothermal amplification (LAMP), to detect specific genes in less than 3 h [10–16]. Although this method has been applied in the field of microbiology for detection and identification of *Mycobacterium* sp. [17], hepatitis B virus [14,18], and *Candida* sp. and *Aspergillus* sp. (Fujisaki, personal communication), the technique has not been applied on *P. brasiliensis*. In the present study, we used the LAMP method to detect the *gp43* gene of *P. brasiliensis*. This gene encodes a 43-kDa glycoprotein that is the major antigen of the fungus [19].

* Corresponding author. Tel.: +81-43-226-2786; fax: +81-43-226-2486.
E-mail address: aya1@faculty.chiba-u.jp (A. Sano).

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2. Materials and methods

2.1. Isolates and DNA extraction

Twenty-two clinical isolates (IFM 41620, 41621, 41622, 41623, 41624, 41625, 41626, 41628, 41629, 41630, 41631, 41632, 41633, 46215, 46240, 46464, 46465, 46466, 46467, 46468, 46470, and 46930) and seven nine-banded armadillo (*Dasypus novemcinctus*)-derived (IFM 46463, 47183, 47185, 47195, 47219, 47228, and 47247) isolates of *P. brasiliensis* were evaluated. Yeast-form cells harvested on 1.0% glucose added Difco™ brain heart infusion agar (Becton Dickinson Microbiology Systems, Sparks, MD, USA) slants at 35 °C for seven days were used. Approximately 5×10^8 yeast-form cells were suspended in distilled water (DW) and washed three times with DW, and homogenized in a 1.5 ml volume plastic homogenizer. DNA was extracted with the Gen Toru Kun for yeast (Dr. GenTLE™ for yeast) kit (TAKARA BIO INC., Otsu, Shiga, Japan). Isolates of *Coccidioides immitis* (IFM 50993), *Histoplasma capsulatum* (IFM 41329), *Blastomyces dermatitidis* (IFM 41316), *Sporothrix schenckii* (IFM 47068), *Penicillium marneffei* (IFM 41708), *Candida albicans* (IFM 5740), and *Cryptococcus neoformans* (IFM 5830) cultured on Difco potato dextrose agar (Becton Dickinson Microbiology Systems) at 25 °C for 7–60 days were used as negative controls. The fungal cells of *C. immitis* were fixed with 70% ethanol overnight, and the DNA was extracted by the kit (Dr. GenTLE™ for yeast, TAKARA BIO INC.). The final concentrations of DNA were adjusted from 10 to 20 ng/μl. DNA extracted from a paraffin-embedded tissue sample of PCM with a DEXPAT® kit (TAKARA BIO INC.) was also used in a LAMP assay.

2.2. Detection of gp43 by PCR

A total volume of 25 μl was used for all PCR reactions. Fifty nanograms per millilitre of DNA extracts were added to 2.5 μl of 10× Ex Taq™ buffer in the kit (Ex Taq™, TAKARA BIO INC.) containing 4.5 mM $MgSO_4$, 2 μl (2.5 mM each) dNTP mixture in the kit (Ex Taq™, TAKARA BIO INC.), 2 μl each 10 pM primer set of F3 5′-TCA CGT CGC ATC TCA CAT TG-3′ encoding from 391st to 410th and B3 5′-AAG CGC CTT GTC CAA ATA GTC GA-3′ designed from the complementary sequence from 718th to 740th correspondent to *gp43* sequence at GenBank U26160 and 0.0625 μl (5 units/μl) TaKaRa Ex Taq™ polymerase in the kit (Ex Taq™, TAKARA BIO INC.). Reaction mixtures were subjected to denaturation at 94 °C for 1 min, followed by 30 cycles of amplification at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min and a final extension at 72 °C for 10 min, in a PCR Thermal Cycler MP (TAKARA BIO INC.). PCR products were separated by electrophoresis on 1.0% agarose gels in TAE buffer (40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA), stained with ethidium bromide, and visualized by UV transillumination. DNA bands obtained from the PCR were processed for direct sequencing with ABI Prism 3100 (Applied Biosystems, Foster City, CA., USA) to confirm the sequence of *gp43* [6].

2.3. LAMP method

LAMP, which stands for Loop-Mediated Isothermal Amplification, a novel DNA amplification method developed by Eiken Chemical Co., Ltd. (Tokyo, Japan) has the potential to replace PCR because of its simplicity, rapidity, specificity and cost-effectiveness. The method is characterized by its use of four different primers specifically designed to recognize six distinct regions on the target gene and its process being performed at a constant temperature using a strand displacement reaction. Amplification and detection of gene can be completed in a single step, by incubating the mixture of gene sample, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (in the region of 65 °C). It provides high amplification efficiency, with DNA being amplified 10^9–10^{10} times in 15–60 min. Because of its high specificity, the presence of the target gene sequence can easily be detected just by judging presence of amplified products (http://loopamp.eiken.co.jp/e/tech/commentary/lamp.htm).

Briefly, the LAMP method used in the present study detects the *gp43* gene with a combination of F3, B3, FIP, and BIP primers designed from the partial sequence of *gp43* (GenBank accession number U26160) by a registration system primer designing website (FUJITSU Ltd., Tokyo, Japan: ''LAMP PIMER EXPLORER'' website 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, used in the species specific forward primer; B3, 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 to 445th and the complementary sequence of 464th to 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. The annealing sites of the primers are shown in Fig. 1. One microlitre of 10 ng/ml DNA template and 40 pmol each of the FIP and BIP primers and 5 pmol each of the F3 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 60 min. The reaction was stopped by heating the mixture at 80 °C for 2 min to inactivate the enzyme

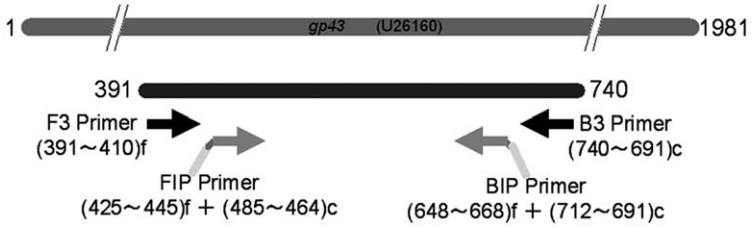

Fig. 1. Primer map for the LAMP method of detecting *gp43* gene of *P. brasiliensis*.

of LAMP amplification. Detection limits of the LAMP method were evaluated with serial dilutions of DNA from isolate IFM 46930. In addition, time-dependent increases in levels of DNA products by LAMP were monitored by real-time-PCR (Rotor-Gene, RG2000, NIPPN/Techno Cluster, Inc., Tokyo, Japan) for as long as 70 min at 63 °C with *P. brasiliensis* isolates IFM 41630 and IFM 46215. As the position control attached with the kit and a negative control consisted of DW and other fungal DNAs, *C. immitis*, *H. capsulatum*, *B. dermatitidis*, *S. schencki*, *P. marneffei*, *C. albicans*, and *Cr. neoformans* were used.

In addition, DNA extracted from a paraffin-embedded tissue sample was reacted at 63 °C for 60 and 120 min.

3. Results

The PCR products amplifed with the primer set; F3 and B3 showed species specificity for *P. brasiliensis*. Other related species, such as *C. immitis*, *H. capsulatum*, and *B. dermatitidis* were negative nor important pathogenic fungi; *S. schenckii*, *P. marneffei*, *C. albicans*, and *Cr. neoformans* (Fig. 2). The detection limit of the PCR was 100 fg of fungal genomic DNA. All partial sequences of *gp43* detected by the primer set were coincident to the accession numbers of GenBank; AB047815, AB047814, AB047813, AB047705, AB047704, AB047703, AB047702, AB047701, AB047700, AB047699, AB047698, AB047697, AB047696, AB047695, AB047694, AB047693, AB047692, AB047691, and AB047690.

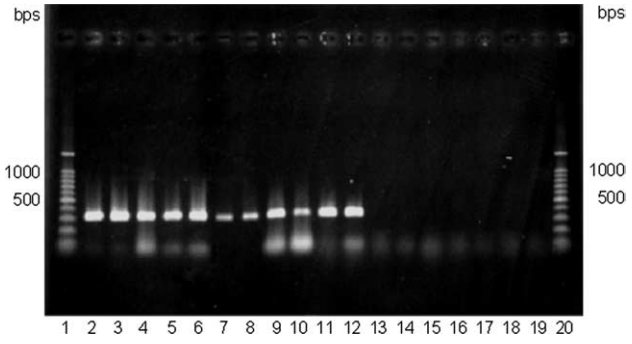

Fig. 2. Amplification of the *gp43* gene by PCR with primers F3 and B3. All fungal DNAs from *B. brasiliensis* are positive. Lane 1, marker; 2, IFM 46467; 3, IFM 46468; 4, IFM 46470; 5, IFM 46930; 6, IFM 46463; 7, IFM 47183; 8, IFM 47185; 9, IFM 47195; 10, IFM 47247; 11, IFM 47219; 12, IFM 47228; 13, *C. albicans*; 14, *H. capsulatum*; 15, *B. dermatitidis*; 16, *P. marneffei*; 17, *S. schenckii*; 18, *C. neoformans*; 19, *C. immitis*; and 20, marker.

The specific DNA banding pattern of *P. brasiliensis* was detected in the clinical and nine-banded armadillo-derived isolates by LAMP. No DNA band was observed in negative control isolates of *C. immitis*, *H. capsulatum*, *B. dermatitidis*, *S. schenckii*, *P. marneffei*, *C. albicans*, and *Cr. neoformans* (Fig. 3a). The incubation procedure at 63 °C for 60 min was not sufficient for detection of *gp43* from DNA extracted from paraffin-embedded tissue sample infected with PCM. The DNA from a paraffin-embedded tissue yielded the same ladder band yielded by fungal DNAs via LAMP at 63 °C for 120 min (Fig. 3b). The detection limit of LAMP for *gp43* was also 100 fg of fungal genomic DNA.

The LAMP reaction reached a plateau after incubation at 63 °C for 45 min, so far, as monitored by real-time-PCR (Fig. 4). The positive control provided with the kit reached a plateau at 15 min, and the negative one did not show increase of fluorescence level. DNAs from other fungal species did not increase the fluorescence level (data not shown). The LAMP reaction of DNA from isolate IFM 46215 reached a plateau at 63 °C for 45 min and those of IFM 41622 was 50 min.

4. Discussion

The present study confirmed the reliability of LAMP for detecting the *gp43* gene of *P. brasiliensis*. The LAMP method provides for more rapid detection of *gp43* than nested PCR. LAMP required only 3 h from DNA extraction to identification, whereas nested PCR required 12 h.

LAMP is also advantageous because it can be applied to clinical materials such as paraffin-embedded tissue samples for retrospective study. Even in paraffin-embedded tissue samples, the time required for diagnosis was only a few hours. The LAMP method is not only

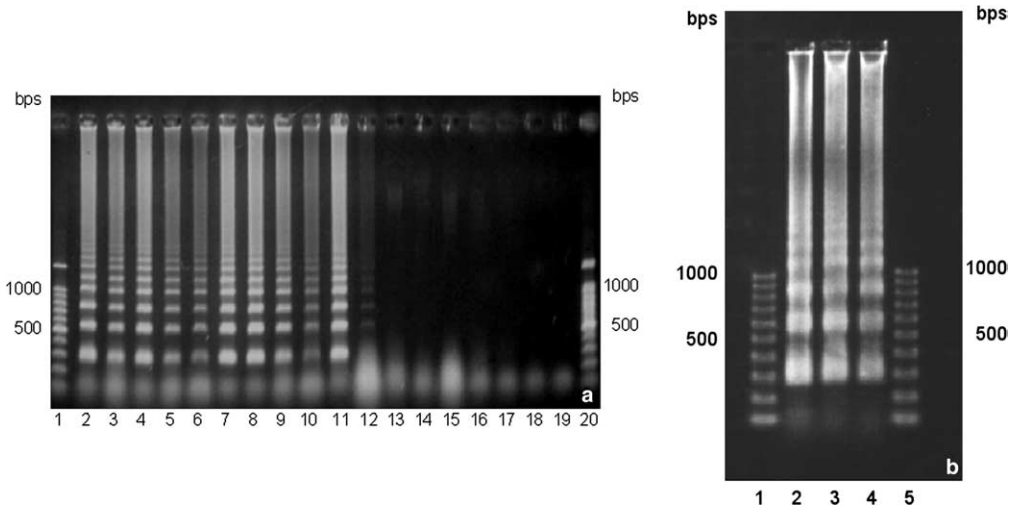

Fig. 3. The specific DNA banding patterns for the *gp43* gene of *P. brasiliensis* by LAMP. (a) All fungal DNAs from *B. brasiliensis* are positive. Lane 1, marker; 2, IFM 46467; 3, IFM 46468; 4, IFM 46470; 5, IFM 46930; 6, IFM 46463; 7, IFM 47183; 8, IFM 47185; 9, IFM 47195; 10, IFM 47247; 11, IFM 47219; 12, IFM 47228; 13, *C. albicans*; 14, *H. capsulatum*; 15, *B. dermatitidis*; 16, *P. marneffei*; 17, *S. schenckii*; 18, *C. neoformans*; 19, *C. immitis*; and 20, marker. (b) The specific DNA banding patterns for the *gp43* gene of *P. brasiliensis* of a case of PCM by LAMP. Lane 1, marker; 2, DNA extracted from paraffin-embedded tissue sample of a case of PCM; 3, IFM 46930; 4, IFM 46215; and 5, marker.

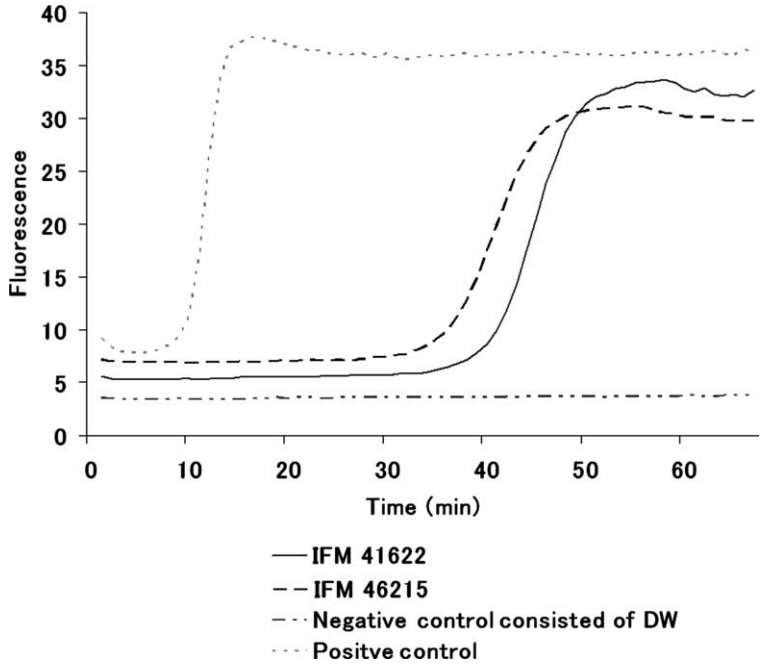

Fig. 4. LAMP reaction monitored by real-time-PCR. The negative control with other fungal DNAs, *C. immitis*, *H. capsulatum*, *B. dermatitidis*, *S. schenckii*, *P. marneffei*, *C. albicans*, and *Cr. neoformans* were as the same as DW.

convenient for identification of *P. brasiliensis*, but also for diagnosis of PCM. Identification of *P. brasiliensis* and diagnosis of PCM outside of endemic areas, including Japan, have been problematic. Patients are sometimes misdiagnosed as having a malignant tumor because of a shadow on the chest X-ray and granulomatous inflammation of infected tissue. Therefore, most PCM of patients in Japan are being diagnosed on the basis of histopathological findings [2]. The LAMP method could be applied for diagnosis in such cases without isolation of the fungus. We tested only one clinical sample of PCM in the present study; thus, further studies are needed before the method can be applied to other clinical materials such as blood, urine, sputum and biopsy specimens.

Application of real-time-PCR to the LAMP method should shorten the time to obtain the results with in a couple of hours, because electrophoresis is not required.

While analysis of LAMP amplification products by agarose gel electrophoresis takes approximately 3 h, LAMP in connection with real-time-PCR takes only 2 h. According to the manufacturer's protocol, LAMP products can be detected by optical density. In addition, the reaction does not require a special thermocycler system, meaning the method will be useful in field hospitals.

The LAMP method will be applicable to clinical identification of fungi and diagnosis of fungal diseases caused by level 3 biohazards, such as coccidioidomycosis, histoplasmosis, blastomycosis and infection of *P. marneffei*, which generally require dangerous and time-consuming culturing procedures. We expect the LAMP to become useful for diagnosing not only PCM and other fungal diseases but also other serious diseases.

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References

[1] Restrepo, A.M. (1985) The ecology of *Paracoccidioides brasiliensis*: a puzzle still unsolved. J. Med. Vet. Mycol. 23, 323–334.
[2] Fujio, J., Nishimura, K. and Miyaji, M. (1999) Epidemiological survey of the imported mycoses in Japan. Jpn. Med. Mycol. 40, 103–109.
[3] Goldani, L.Z. and Sugar, A.M. (1998) Short report: Use of the polymerase chain reaction to detect *Paracoccidioides brasiliensis* in murine paracoccidioidomycosis. Am. J. Trop. Med. Hyg. 58, 152–153.
[4] de Brito, T., Sandhu, G.S., Kline, B.C., Aleff, R.A., Sandoval, M.P., Santos, R.T., Brandão, A.A. and Lacaz, C.S. (1999) In situ hybridization in paracoccidioidomycosis. Med. Mycol. 37, 207–211.
[5] Motoyama, A.B., Venancio, E.J., Brandão, G.O., Petrofeza-Silva, S., Pereira, I.S., Soares, C.M. and Felipe, M.S. (2000) Molecular identification of *Paracoccidioides brasiliensis* by PCR amplification of ribosomal DNA. J. Clin. Microbiol. 38, 3106–3109.
[6] Sano, A., Yokoyama, K., Tamura, M., Mikami, Y., Takahashi, I., Fukushima, K., Miyaji, M. and Nishimura, K. (2001) Detection of gp43 and ITS 1 – 5.8S – ITS 2 ribosomal RNA genes of *Paracoccidioides brasiliensis* in paraffin-embedded tissue. Jpn. J. Med. Mycol. 42, 23–27.
[7] Bialek, R., Ibricevic, Aepinus, C., Najvar, L.K., Forthergill, A.W., Knobloch, J. and Graybill, J.R. (2000) Detection of *Paracoccidioides brasiliensis* in tissue samples by a nested PCR assay. J. Clin. Microbiol. 38, 2940–2942.
[8] Diez, S., Garcia, E.A., Pino, P.A., Botero, S., Corredor, G.G., Peralta, L.A., Castaño, J.H., Restrepo, A. and McEwen, J.G. (1999) PCR with *Paracoccidioides brasiliensis* specific primers: potential use in ecological studies. Rev. Inst. Med. Trop. São Paulo 41, 351–357.
[9] Gomes, G.M., Cisalpino, P.S., Taborda, C.P. and de Camargo, Z.P. (2000) PCR for diagnosis of paracoccidioidomycosis. J. Clin. Microbiol. 38, 3478–3480.
[10] Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. (2000) Loop-mediated isothermal amplification of DNA. Nucl. Acids Res. 28, E63.
[11] Takano, H. and Notomi, T. (2001) An outline of LAMP method: simple and rapid gene amplification method. ACCP 7, 62–68.
[12] Notomi, T. and Hase, T. (2001) The principle and application of a novel gene amplification method (LAMP Method). BioIndustry 18, 15–23.
[13] Kanda, H. and Notomi, T. (2001) A novel gene amplification method. Pharmacia 37, 889–893.
[14] Nagamine, K. and Watanabe, K. (2001) Loop-mediated isothermal amplification reaction using a nondenatured template. Clin. Chem. 47, 1742–1743.
[15] Mori, Y., Nagamine, K., Tomita, N. and Notomi, T. (2001) Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289, 150–154.
[16] Nagamine, K., Kuzuhara, Y. and Notomi, T. (2002) Isolation of single-stranded DNA from loop-mediated isothermal amplification products. Biochem. Biophys. Res. Commun. 290, 1195–1198.
[17] Iwamoto, T., Sonobe, T. and Hayashi, K. (2003) Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. J. Clin. Microbiol. 41, 2616–2622.
[18] Nagamine, K., Hase, T. and Notomi, T. (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell Probes 16, 223–229.
[19] Cisalpino, P.S., Puccia, R., Yamauchi, L.M., Cano, M.I., da Silveira, J.F. and Travassos, L.R. (1996) Cloning, characterization, and epiotope expression of the major diagnostic antigen of *Paracoccidioides brasiliensis*. J. Biol. Chem. 271, 4553–4560.