

Short communication

## Utility of Real-time PCR for the detection of *Paracoccidioides brasiliensis* DNA in the diagnosis of imported paracoccidioidomycosis

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An increase in immigration from endemic regions has resulted in a number of cases of paracoccidioidomycosis (PCM) being imported into Spain. A molecular diagnostic technique based on real-time PCR was developed for the detection of *Paracoccidioides brasiliensis* DNA in both culture and patients' clinical samples. A Molecular Beacon probe was used, labelled with FAM and directed at the ITS1 region of ribosomal DNA. The detection limit of the technique developed was 1 fg of fungal DNA per µl of sample. This procedure proved to be very reproducible and specific. The technique was tested with cultures of 12 clinical strains and on samples from two patients with proven PCM. Real-time PCR was positive for all the culture strains, as well as those from both patients. By samples, the technique was positive in sputum and tissue biopsies but less useful on blood samples. Samples were analyzed several months after patient treatment, detecting a small amount of fungal DNA in one respiratory sample. This technique of real-time PCR is a sensitive method for rapid diagnosis of paracoccidioidomycosis and could serve to monitor patients after treatment has begun.

**Keywords** Real-time PCR, paracoccidioidomycosis, endemic mycoses

### Introduction

Although paracoccidioidomycosis (PCM) is an infrequent disease in Europe several cases have been described in recent years [1–4] in patients from endemic regions or travellers who have visited these areas [5]. The increase in the immigration of individuals from South America to Spain has caused a rise in the prevalence of tropical mycoses [6,7], including PCM [1,8,9]. In non-endemic areas, diagnosis of PCM is hampered by lack of experience of the clinical staff, the long latent period of the disease and

its resemblance to tuberculosis, sarcoidosis and squamous cell carcinoma [5].

A new Real-time PCR-based assay was designed to detect DNA of *Paracoccidioides brasiliensis* in culture and in clinical samples. The development of this type of technique for identifying the fungus from a culture can significantly reduce the risk of handling cultures and can shorten the time needed to identify the fungus. In clinical samples these techniques can also be used as a method of early diagnosis and for monitoring response to treatment.

### Material and methods

#### Microorganisms

A total of 12 clinical strains of *P. brasiliensis* were used. Five were isolated by the Mycology Service Collection at the Spanish National Centre of Microbiology from PCM

Received 3 April 2008; Final revision received 26 November 2008; Accepted 24 December 2008

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patients who had immigrated to Spain. The other seven isolates were obtained from the Evandro Chagas Institute of Research, Brazil. For the specificity studies, the following fungi were employed; *Coccidioides immitis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, *Fusarium verticillioides*, *Fusarium oxysporum*, *Scedosporium prolificans* and *Scedosporium apiospermum*. Human and mouse DNA were also included in the experiments (Promega, Madrid, Spain).

#### Extraction of DNA from strains

The extraction of nucleic acids followed the usual procedures [10]. In the case of *P. brasiliensis*, *C. immitis*, *H. capsulatum* and *B. dermatitidis*, extraction was undertaken using the method described by Buitrago et al. [7]. For rapid identification in culture, a small amount of mycelium was taken and DNA was extracted with the Wizard SV Genomic DNA Purification Kit (Promega, Madrid, Spain) following manufacturer's instructions.

#### Design of primers and probe

The Beacon Designer programme, version 5.0. was used. The primers and the probe were designed in the ITS1 region of the ribosomal DNA. The direct primer (OliPbMB1) was 5'-ACCCTGTCTATTCTACC-3' and the reverse (OliPbMB2) was 5'-TTACTGATTATGATAGGTCTC-3', amplifying a fragment of 144 base pairs. The probe (PbMB1) had a sequence 5'-FAM CGCGATCGCCGGGACACCGTTGAAATCGCG BHQ1-3' (Sigma-Genosys, Madrid, Spain).

#### Description of the Real-time PCR technique

The PCR reactions were undertaken in a LC480 unit (Roche Applied Science) and in a Chromo4 unit (MJ Research, Bio-Rad). The Kit SensiMix (dT) (Quantace, Ecogen, Spain) was used following the manufacturer's instructions. Real-time reactions contained a final volume of 20 µl with 3 mM of MgCl<sub>2</sub>, 0.5 µM of each primer and 0.6 µM of probe. Next, 2 µl of DNA was added to the PCR mix. As regards the conditions of the real-time PCR, pre-incubation occurred at 95°C for 10 min. Next followed 45 denaturation cycles (15 s at 95°C, 30 s at 56°C and 5 s at 72°C). Each experiment included negative controls and one positive control (20 pg of DNA from the CNM-CM2908 strain).

#### Study of the reproducibility, sensitivity and specificity of the in vitro technique

To study reproducibility, as well as quantifying the DNA, different dilutions of *P. brasiliensis* DNA were

employed (from 10 ng to 1 fg DNA/µl) and six regression lines representing the logarithms of the DNA concentrations against the crossing points (Ct) were constructed. Reproducibility was obtained by calculating the coefficients of variation of the Ct for each of the concentrations of DNA used. Specificity was determined including 2 ng of DNA of the strains mentioned in the *Microorganisms* section, as well as human and mouse DNA.

#### Use of the technique in cultures

Two µl of DNA of the 12 strains were used in the Real-time PCR.

#### Use of the technique in clinical samples

The utility of Real-time PCR for diagnosis of clinical PCM was evaluated through the use of samples from two confirmed cases of the disease. The Case 1 was an Ecuadorian male, 67 years old, with asthenia, sporadic fever and pustulous lesions which had developed over a month in the frontal region and extremities, and which ulcerated leaving an indurated edge. The patient's clinical history revealed that he had been under observation for one year after presenting a radiograph of thorax with bilateral central patched infiltrated alveoli which had not produced any respiratory symptoms and had been diagnosed with PCM in April, 2006. The Case 2 was a Spanish male patient, 57 years of age, who had worked as a missionary for 10 years in Venezuela. For a period of six months the patient presented with episodes of coughing lasting several days, these becoming increasingly frequent and prolonged, with the later appearance of yellowish expectoration, on occasions hemoptoic. The cough was accompanied by fever with no other signs or symptoms. The patient was diagnosed with PCM in January 2007.

Samples of serum, blood, sputum and paraffined skin biopsy from case 1, taken at the time of diagnosis (April 2006), plus a sputum and blood sample, were received in February 2007 for follow-up. For case 2, samples of serum, blood and sputum were received in January 2007, and further samples of serum and sputum were received again two months later, in March of the same year.

Extraction of DNA from clinical samples (serum, blood, sputum) used the QAamp DNA Mini Kit (Qiagen). Purified DNA was eluted in 50 µl of elution buffer. Sputum samples were homogenized with a vortex and 200 µl were employed to perform the DNA extraction. Smaller samples were adjusted to 200 µl with Phosphate Buffered Saline (PBS). The biopsy in paraffin was deparaffined by one lavage with 1.2 ml of xylene followed by two lavages with 1.2 ml of ethanol (96–100%). After incubating the tissue at 37°C to evaporate the remains of the ethanol, DNA was

extracted using the QiAmpTissue DNA Mini Kit (Qiagen). Two  $\mu\text{l}$  of DNA extracted from each sample was used for each real-time PCR reaction. In the case of the serum, we attempted to increase the amount of the sample using 4 and 6  $\mu\text{l}$  and obtained the same result. Table 1 shows the samples analyzed and results obtained.

The fungal load was quantified using the straight-line of regression described above. The amplified products were sequenced in order to verify that they were *P. brasiliensis*. The experiments were run twice in the two different units.

To assess the specificity of the PCR technique, we analyzed 10 samples from patients with confirmed histoplasmosis, 10 samples from patients with confirmed aspergillosis, 30 samples from neutropenic patients with no fungal infection and five samples, including blood, serum and respiratory samples from healthy individuals from endemic regions.

#### Technique of Immunodiffusion

Immunodiffusion (ID Fungal Antibody System, Immuno-Mycologies, Leti Laboratorios, Madrid, Spain) was also carried out on the serum samples to determine the presence of antibodies for *P. brasiliensis*. The specific antigen used was gp43.

## Results

#### Standardization in vitro of the Real-time PCR technique

The detection limit of the technique was set between 10 and 1  $\text{fg}/\mu\text{l}$  of sample. Reproducible DNA was detected from 10 ng to 10 fg with CVs on average of 3% in the six straight lines. With smaller amounts of DNA, linearity was lost and, moreover, coefficients of variation were higher.

As regards the test's specificity, no amplification signal was detected when nucleic acids from other fungal species nor when human or mouse DNA was analyzed.

#### Results of Real-time PCR in cultures

Real-time PCR was run with two  $\mu\text{l}$  of DNA extracted from the 12 strains of *P. brasiliensis*. In all cases the technique was positive, detecting an amplification curve. The fragments amplified were sequenced confirming their identity as DNA from *P. brasiliensis*. The *C<sub>t</sub>*s varied between cycles 14 and 27. The experiment was undertaken in duplicate on different days.

#### Results of Real-time PCR in clinical samples

We received three samples of serum, three of blood, four of sputum and one paraffined skin biopsy. Samples received at the start of the infection were two serum, two blood, two sputum and one paraffined skin biopsy. The PCR was positive for both patients. By samples, the technique was positive in all the sputum samples analyzed, in the paraffined biopsy, and in only one of the two blood samples. The Real-time PCR was negative in 100% of the serum samples analyzed. In the samples received after the patients had been treated, the PCR was positive in one sputum sample of one of the two patients. In the case of the amount of DNA obtained, the mean for the sputum samples at time of diagnosis was 491.41 fg per  $\mu\text{l}$  of sample and in the skin biopsy the mean was 92.08  $\text{fg}/\mu\text{l}$ . However, only 3.25  $\text{fg}/\mu\text{l}$  was obtained from blood sample CS-249. When the post-treatment samples were received and analyzed, the amount of DNA detected in the sputum was found to be 9.86  $\text{fg}/\mu\text{l}$  but no DNA was detected in serum or blood.

**Table 1** Microbiological results of samples from patients suffering from paracoccidioidomycosis

Patient	Samples	Date	RT-PCR result	DNA concentration ( $\text{fg}/\mu\text{l}$ )	Immunodiffusion result
1	Serum (CS-248)	April 2006	Negative	–	Light Positive
	Blood (CS-249)		Positive	3.25	ND
	Sputum (CS-255)		Positive	390.34	ND
	Skin Biopsy (CS-256)		Positive	92.08	ND
	Sputum (CS-420)	February 2007	Negative	–	ND
	Blood (CS-421)		Negative	–	ND
2	Serum (CS-404)	January 2007	Negative	–	Positive
	Blood (CS-405)		Negative	–	ND
	Sputum (CS-406)		Positive	592.49	ND
	Serum (CS-456)	March 2007	Negative	–	Positive
	Sputum (CS-457)		Positive	9.86	ND

ND: Not done

–: Not detected

Regarding the technique's results for samples from patients with confirmed histoplasmosis, confirmed aspergillus, neutropenic patients with no fungal infection, and healthy individuals from endemic regions, the PCR was negative in all samples analyzed, thus specificity was also 100% in clinical samples.

#### Result of Immunodiffusion in serums

Results were positive for the three serums analyzed although one (CS-248) gave a very weak positive result at the onset of the infection.

### Discussion

The technique developed was evaluated with a total of 12 strains of *P. brasiliensis* and also with samples from the two patients with confirmed PCM. All the strains of *P. brasiliensis* showed a positive result, whereas none of the strains from the other species included in the trials as controls ever gave a positive signal in the real-time PCR.

In the patient samples, the result was positive for both patients, detecting the DNA of the fungus, although the same results were not obtained for all the samples. Due to the small number of samples, no statistical analysis could be done, although the respiratory samples appeared to be more useful than blood samples, with 100% of sensitivity in the first case as opposed to 50% for blood. The serum samples were negative in spite of increasing the sample amount to 6 µl. On the other hand, the amount of DNA in blood was significantly lower than that of respiratory secretions or skin biopsy. This results are in agreement with the clinical status of the patients. Infection in both cases was not disseminated so the amount of fungal DNA circulating by blood cells should be very low.

When samples from the same patients were analyzed months after initiating treatment, a negative result was obtained in one patient, while in the other a small amount of DNA was still detected in one sputum sample, highlighting the fact that antifungal treatment should not be suspended.

A greater number of clinical samples would thus need to be analyzed, however preliminary results from studies underway enable us to conclude that this technique is both sensitive and specific. In addition, it may have an

application as a method of early identification of the fungus in culture and in clinical samples of PCM.

### Acknowledgements

This work was supported by Research Projects from Instituto de Salud Carlos III (MPY 1279/05), and from RED Española de Investigación en Patología Infecciosa (REIPI, MPY 1022/07).

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

### Note

This paper was presented in part at the 47th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, 2007.

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