SHORT REPORT: USE OF THE POLYMERASE CHAIN REACTION TO DETECT PARACOCCIDIOIDES BRASILIENSIS IN MURINE PARACOCCIDIOIDOMYCOSIS

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Abstract. The polymerase chain reaction (PCR) was used to detect the presence of Paracoccidioides brasiliensis in a murine model of disseminated paracoccidioidomycosis. Using a previously identified P. brasiliensis-specific DNA sequence, P. brasiliensis DNA was detected in serum of five experimentally infected mice. The PCR method was able to detect as little as 10 pg of P. brasiliensis DNA in serum, and it was more sensitive than blood culture isolation (five of five were PCR positive versus two of five blood culture positive). There were no amplified fragments in serum from three noninfected control mice. Lung colony counts were similar in all infected mice and reflected a similar degree of P. brasiliensis infection at the time the samples were drawn. The relatively short processing time for the PCR, when compared with culture, its sensitivity, and the possibility of using serum samples for analysis, are important factors favoring this method for the diagnosis of paracoccidioidomycosis. Future studies should include the detection of P. brasiliensis in patients with different clinical forms of paracoccidioidomycosis.

Paracoccidioides brasiliensis is the etiologic agent of paracoccidioidomycosis, the most prevalent systemic mycosis in Latin America. Paracoccidioidomycosis is a disorder that primarily involves the lungs and then disseminates to other organs by lymphatic and hematogenous routes. Secondary lesions appear frequently in the mucous membranes, skin, lymph nodes, and adrenal glands. Diagnosis currently relies on direct microscopic examination, the ability to grow the fungus in the laboratory, and serologic tests.

The polymerase chain reaction (PCR) has been found to be a sensitive, specific, and rapid method for the detection of variety of microorganisms, and it could be a useful diagnostic tool in diagnosing paracoccidioidomycosis.

In the present study, we have evaluated a PCR method to detect P. brasiliensis DNA in serum in a established murine model of disseminated paracoccidioidomycosis. We have compared the sensitivity of detection of P. brasiliensis DNA in serum with blood cultures and colony counts in the lungs.

Male, eight-week-old, ICR mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). Groups of five mice were housed in filter-topped cages and fed standard mouse chow and water ad libitum. A cohort of three additional mice were added as a control group for the study.

Paracoccidioides brasiliensis (Pb 18) was maintained on Sabouraud dextrose agar slants at 4°C. A large loopful of organisms was suspended in fresh Sabouraud dextrose broth and incubated for 96 hr at 37°C. The P. brasiliensis yeast cells were harvested and washed three times in sterile buffered saline (pH 7.4) by centrifugation. Cells were counted in a hemacytometer and adjusted to a concentration of 5 x 10^7/ml. The number of yeast cells administered to the mice was determined by plating the same inoculum on Sabouraud dextrose agar plates and incubating the cultures for five days at 37°C.

Mice were injected by the intraperitoneal route with 1 ml of sterile saline saline containing 5.8 x 10^6 yeast cells. The PCR was performed with serum samples (0.5 ml) drawn eight weeks after the mice were infected. The P. brasiliensis yeast DNA was extracted from serum samples by adding proteinase K, sodium dodecyl sulfate, and Triton X-100 to 0.5 ml of serum to final concentrations of 15 μg/ml, 1%, and 1%, respectively, followed by incubating the mixture at 37°C for 60 min on a shaker. The samples were then boiled for 5 min, cooled to 40°C, extracted with phenol-chloroform-isooamyl alcohol, and precipitated with ethanol. The P. brasiliensis DNA was amplified by PCR by using primers derived from a specific cloned DNA fragment from P. brasiliensis; 5'-TCGTATCCCTCATGA-3 (primer 1) and 5'-AAGAGTCATCCCTCGC-3 (primer 2). The PCR was carried out in a total volume of 50 μl containing 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl2, 0.4 mM of each nucleotide, 1.25 U of AmpliTaq DNA polymerase (all reagents were obtained from Perkin-Elmer Cetus (Norwalk, CT). The DNA amplification was initiated with a 5-min denaturation step at 94°C, followed by 35 cycles at 94°C for 60 sec, 42°C for 60 sec, 72°C for 2 min, and a final 10-min extension step at 72°C. The amplified DNA (10 μl) was analyzed by electrophoresis on 4% agarose gels in 1× TBE buffer (90 mM Tris-Borate, 2 mM EDTA) at 120 V for 30 min. Gels were stained with ethidium bromide. Samples containing a fragment of 62 basepairs were regarded as positive. To determine the level of sensitivity of this PCR assay, P. brasiliensis (Pb18) DNA as a positive control was diluted in water and the PCRs were performed with 1 ng, 100 pg, 10 pg, and 1 pg of genomic DNA. Our PCR method was able to detect as little as 10 pg of P. brasiliensis DNA. Additionally, 1 ml of blood from each animal was inoculated in a 1.5-ml isolator blood culture tube (Canpole Laboratories, Cranbury, NJ), a non-automated lysis-based method. After centrifugation, the isolator concentrate was processed according to the manufacturer's instructions. The isolator sediment was inoculated

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Serum PCR</th>
<th>Blood culture</th>
<th>Lung culture (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive (1.8 x 10^3)</td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive (4.2 x 10^3)</td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive (6.3 x 10^3)</td>
</tr>
<tr>
<td>4</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive (3.2 x 10^3)</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive (2.6 x 10^3)</td>
</tr>
</tbody>
</table>

*PCR = polymerase chain reaction; CFU = colony-forming units.

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TABLE 1
Analysis of blood samples and lung cultures from mice inoculated by the intraperitoneal route with Paracoccidioides brasiliensis
onto Sabouraud dextrose agar plates and incubated for 30 days at 37°C, at which time the number of colonies on the plates were counted. Mice were killed after eight weeks of infection and lungs were removed. The lungs were homogenized in 500 μl of buffered saline, cultured on Sabouraud dextrose agar plates, and incubated at 37°C for 30 days.

Results of lung cultures are presented in Table 1 and confirm a similar degree of *P. brasiliensis* infection in all infected mice. Isolator blood cultures were positive in two mice (Table 1). The *P. brasiliensis* DNA was amplified from serum of all five infected mice (Figure 1). In contrast, there were no amplified products from serum of all three noninfected control mice.

The PCR assay used in this study was useful in detecting the presence of *P. brasiliensis* DNA in mice experimentally infected with this dimorphic fungus. To date, this is the first report describing a PCR method for detecting *P. brasiliensis* in an experimental model. In addition to obviating the need for invasive biopsies to document the presence of *P. brasiliensis*, this PCR method uses serum, thereby obviating the need for tedious isolation procedures for DNA from whole blood. Moreover, our PCR method had better sensitivity and faster results than blood cultures in detecting *P. brasiliensis* (five of five versus two of five positive). Although we showed that the PCR-generated DNA sequence is not present in other fungi that might cause disease, which could be confused with paracoccidioidomycosis, sera from mice with other fungal infections will need to be evaluated to ascertain the specificity of this method of diagnosis of this disease.

Further evaluation of the PCR with serum of patients with different clinical forms of paracoccidioidomycosis is needed to assess the true value of this new diagnostic approach.

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