Posttreatment Follow-Up of Brucellosis by PCR Assay

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In order to evaluate the usefulness of a peripheral blood PCR assay in the posttreatment follow-up of brucellosis, a cohort of 30 patients was studied by means of blood cultures, rose Bengal, seroagglutination, Coombs’ antibrucella tests, and PCR assay at the time of diagnosis, at the end of treatment, and 2, 4, and 6 months later. Of the 29 patients whose PCR assays were initially positive, 28 (96.5%) were negative at the conclusion of the treatment. PCR was positive for the two patients who had relapses and negative for another four who had suspected but unconfirmed relapses. PCR was negative for 98.3% of the follow-up samples from those patients who had a favorable evolution. In conclusion, PCR appears to be a very useful technique, not only for the initial diagnosis of the disease, but also for posttreatment follow-up and the early detection of relapses.

Brucellosis is a world-wide zoonosis transmissible to humans, in whom it has a high degree of morbidity (14, 26). Brucella organisms are able to survive and even multiply within cells of the mononuclear-phagocytic system, thus explaining the tendency of the disease to have a prolonged clinical course and relapses (9, 18, 20). Even with the correct treatment, the incidence of relapses in brucellosis remains high, ranging from 4 to 41% of patients in the largest series reported to date (3, 7, 17).

The clinical picture of brucellosis is very heterogeneous and nonspecific (8), and the clinical manifestations of relapsed brucellosis are milder, overlapping, and nonspecific, the hematological and biochemical changes being even more subtle than in the initial infection. Diagnosis of brucellosis relapses is therefore generally difficult, and microbiological techniques are often required for confirmation. However, the yield of blood cultures in relapses is no higher than 60 to 70% (2, 23), and the value of serological diagnosis in this situation is limited (20).

PCR has been shown to be a more sensitive technique than blood cultures and more specific than conventional serological tests for the diagnosis of infection with Brucella melitensis (21). We therefore evaluated the usefulness of a PCR-based assay in the posttreatment follow-up and the early diagnosis of relapses in patients with brucellosis.

Patient population. From January 1997 to March 1998, we studied a cohort of 30 patients with brucellosis. Twenty-eight were diagnosed and treated in the Infectious Diseases Unit of “Carlos Haya” Hospital in Malaga, Spain, and the other 2 were treated in the Infectious Diseases Service of “Virgen del Rocío” University Hospital in Seville, Spain. The diagnosis of brucellosis was established according to one of the following criteria: (i) isolation of Brucella spp. in blood or any other body fluid or tissue sample or (ii) the presence of a compatible clinical picture together with the demonstration of specific antibodies at significant titers or seroconversion. Significant titers were considered to be a Wright's seroagglutination titer of ≥1/160 or a Coombs' antibrucella test titer of ≥1/320.

Of the 30 patients included, 16 (53.3%) were men and 14 (46.7%) were women. The mean age of the group was 40.1 ± 14.5 years (range, 17 to 71 years). In 26 patients (86.6%), the brucellosis was the first episode of infection, in 2 (6.6%) it was a reinfection, and in the other 4 (13.3%) it was a relapse. The duration of the symptoms prior to diagnosis was 11.7 ± 22.7 weeks (range, 1 to 102 weeks). In 9 patients (30%), the duration of the symptoms was less than 2 weeks; in eight (26.6%), it was between 2 weeks and 1 month; in another 8 (26.6%), it was between 1 and 3 months; and in the other five (16.6%), it was longer than 3 months. All of the patients had fever during the evolutionary course of the disease. The clinical picture in 17 (56.6%) patients was a nonfocal febrile syndrome, and the other 13 (43.3%) had one or more focal forms (3 with spondylitis, 2 with liver abscess, and 1 each with sacroiliitis, knee arthritis, wrist arthritis, oligoarthritis, hepatitis, splenic abscess, pneumonia, infected ovarian teratoma, or infected renal cyst).

All patients with suspected brucellosis had two or more blood cultures and a serological battery of tests, including the rose Bengal plate agglutination test, Wright’s seroagglutination, and Coombs’ antibrucella test. A 3.5-ml peripheral blood sample was also taken for PCR analysis.

The blood cultures were processed in a BACTEC 9240 (Becton Dickinson Diagnostic Instrument Systems, Towson, Md.) according to the usual techniques. For those cases in which the system failed to detect any growth, the incubation was maintained for 30 days, with blind subcultures performed after 10, 20, and 30 days. Identification of Brucella spp. was made according to standard microbiological techniques (11). All of the strains isolated were sent to the National Brucellosis Reference Laboratory in Valladolid, Spain, for definitive identification and biotyping. The serological tests were performed according to previously described techniques (1, 12, 16).

The diagnosis of brucellosis was established by isolation of Brucella spp. in 22 patients (73.3%), for 21 of whom the isolation was made by blood culture (66.7%) and for 1 (6.7%) of whom the isolation was made with synovial fluid. Diagnosis in the remaining eight (26.7%) patients was clinical and serological. All of the strains isolated were identified as B. melitensis: 18 were biobar 1 (81.8%), 3 were biobar 2 (13.6%), and 1 was biobar 3 (4.5%).
After diagnosis of the brucellosis, 26 (86.7%) patients were treated with doxycycline plus streptomycin sulfate, and 3 (10%) were treated with doxycycline plus rifampin according to internationally accepted treatment regimens (3). The remaining patient was a woman who was 4 weeks pregnant and who was treated with rifampin alone for 3 months. The patients were examined at the end of treatment and after 2, 4, and 6 months, as well as at any intermediate time if relapse was suspected, and at each evaluation, the following tests were performed: blood cultures, rose Bengal, Wright’s seroagglutination, Coombs’ test, and the PCR assay. Therapeutic failure was considered to be the persistence or worsening of the symptoms or signs of the disease after 15 days of treatment in those patients with nonfocal forms of the disease and after 1 month of treatment in patients with focal forms. The criteria for the definition of the different focal forms have been published previously by our group (8). Relapse was considered to be either (i) the existence of a new positive blood culture, (ii) the reappearance of a compatible symptomatology not otherwise explained together with a new increase in the previous serological titers, or (iii) the appearance of a new focal form highly suggestive of brucellosis (e.g., peripheral arthritis, sacroiliitis, orchepididymitis, lymphocytic meningitis, endocarditis, etc.) with persistently high serological titers.

**PCR assay.** Peripheral blood for PCR was collected in sodium citrate and stored at −20°C until processing. PCR was carried out by our previously reported technique (15, 21). Briefly, this consists of amplification of a 223-bp fragment from the gene coding for the synthesis of an immunogenic protein on the external membrane of *Brucella abortus* (BCSP31). This protein, with a molecular mass of 31 kDa, is specific to the genus *Brucella* and is present in all of its biovars. The amplification was performed with the primers B4 (5′-TGG CTC GGT TGC CAA TAT CAA-3′) and B5 (5′-CGC GCT TGC CTT TCA GGT CTG-3′) (5). All tests included positive controls of *B. melitensis* Rev-1 DNA and negative controls containing all of the reaction components except DNA. To detect any possible contamination during the extraction stage of the DNA, all of the PCR assays included control samples from a healthy person. Moreover, to ensure the reliability of the results, all of the samples were processed in duplicate. The test was considered positive if the signal from the amplified product was clearly visible in both samples.

**Results.** A total of 137 PCR assays were done, with a mean of 4.5 ± 1.07 assays per patient. At the moment of diagnosis, the PCR was positive for 29 of the 30 patients (96.6%). One patient with pneumonia still had fever 15 days after starting treatment, at which point an empyema developed. A new set of blood cultures were negative, whereas the PCR remained positive.

The PCR was negative on conclusion of the treatment for 28 of the 29 patients (96.5%) whose PCR was initially positive. The only patient for whom the PCR remained positive at the end of treatment was asymptomatic, and the blood cultures were negative. The patient received no additional treatment and remained asymptomatic, the PCR becoming negative in the following revision and remaining so in all subsequent controls during the follow-up period.

Six patients (20%) had symptoms suggestive of relapse during the follow-up period. Of these, two (6.6%) had a confirmed relapse 2 and 5 months after concluding treatment, and in the other four (13.3%), relapse was eventually ruled out: three because of an alternative diagnosis (one infection with *Rickettsia conorii*, one with sinusitis, and one with a urinary tract infection with hydrocele) and the fourth due to self-limitation of all the symptoms after 5 days with no reappearance during the clinical, serological, and bacteriological 12-month follow-up. Table 1 shows the results of the bacteriological, serological, and PCR tests for the patients with suspected relapse. Both patients with relapses had a positive PCR, but only one had positive blood cultures again, and neither had seroconversion of previous titers. Figure 1 shows the evolution of the PCR in the patient who relapsed and whose blood cultures were negative. The PCR in the two patients with relapses became negative again after concluding the treatment of the relapse and, as with 27 of the remaining 28 patients (96.4%), remained persistently negative during the whole follow-up period.

Just one patient in the group with no relapses (3.6%) had a positive PCR at the 6-month control test. He was a 60-year-old farmer with long-term insulin-dependent diabetes mellitus who had had brucellosis complicated by lumbar spondylitis 9

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**TABLE 1. Evaluation of diagnostic tests for patients with suspected relapse**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Relapse result</th>
<th>Result by:</th>
<th>Titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCR</td>
<td>Blood culture</td>
<td>SAT Pre/Rel</td>
</tr>
<tr>
<td>1</td>
<td>Confirmed</td>
<td>+</td>
<td>640/640</td>
</tr>
<tr>
<td>2</td>
<td>Ruled out</td>
<td>−</td>
<td>640/80</td>
</tr>
<tr>
<td>7</td>
<td>Ruled out</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Ruled out</td>
<td>−</td>
<td>80/0</td>
</tr>
<tr>
<td>13</td>
<td>Confirmed</td>
<td>+</td>
<td>2,560/320</td>
</tr>
<tr>
<td>22</td>
<td>Ruled out</td>
<td>−</td>
<td>80/0</td>
</tr>
</tbody>
</table>

* SAT, seroagglutination; Pre/Rel, prerelapse and relapse.
months previously, requiring surgery and treatment with streptomycin sulfate (1 g intramuscularly per day for 21 days and 100 mg of doxycycline per os twice per day for 3 months). When the PCR became positive again, the patient was asymptomatic and had returned to his usual activities with cattle. He presented no biological signs of active infection, the blood cultures were negative, the serological titers continued to fall compared to those in the prior study, and another examination of the lumbar spine by magnetic resonance imaging showed a favorable evolution of the spondylitis.

**Discussion.** One of the main characteristics of brucellosis is its marked tendency to relapse after conclusion of the treatment (2, 26). This problem is related to the ability of *Brucella* spp. to clade some of the basic mechanisms of the host’s immune system, so that the hallmark of the efficacy of any antibrucella treatment is its capacity to reduce the rate of relapse. Since almost 90% of relapses occur during the 6 months following conclusion of the treatment, strict follow-up is necessary during this period in order to detect any relapse as soon as possible and to provide adequate therapy (3, 7, 13, 22, 24). However, a high proportion of brucellosis patients report nonspecific symptoms after the conclusion of their treatment, and because there are no well-defined criteria for complete recovery from brucellosis, it is often difficult to decide whether these patients are really cured.

In general clinical practice, the posttreatment follow-up of patients with brucellosis includes the appropriate clinical examination together with blood cultures and serological tests. To date, however, the very few studies that have examined the clinical and microbiological profiles of relapses in brucellosis have shown the most effective test for the diagnosis of relapses to be blood cultures, since besides being an irrefutable test of active infection, the blood culture may also be positive in asymptomatic patients. However, the sensitivity of blood cultures in the diagnosis of relapses is not very high, ranging from 50 to 65% in the largest series (2, 13, 23).

Serological methods, although faster and easier to perform, lack specificity, with many studies demonstrating that for a long time after conclusion of treatment, serological titers can remain high or even increase in patients with repeatedly negative blood cultures and no evidence of clinical relapse after a considerable follow-up period (4, 10, 20). Some authors have reported a high seroconversion rate with different serological tests for detecting immunoglobulin G antibodies in relapsed patients (4, 6, 25). Nevertheless, at the time of symptom presentation, only 40% of patients show a significant increase in previous serological titers. In the remaining patients, seroconversion takes place within 3 months after onset of the symptoms of relapse, at which time, these data lose virtually all clinical interest (4, 20).

In this study, 96.4% of the patients had a negative PCR on concluding treatment, a fact which would seem very useful for later control. The PCR for the only patient who remained positive at the end of treatment became negative 1 month later, with no evidence of relapse. This may indicate that due to the extremely high detection capacity of the technique, the PCR might, in a very few cases, amplify the DNA of nonviable bacteria or the remains of DNA present in the circulating mononuclear cells of patients who have concluded successful treatment.

As happens with the first episode of infection, the usefulness of PCR in the diagnosis of relapses appears greater than that of blood cultures. However, it must be taken into account that the number of patients included in a study of these characteristics cannot be very high, and since the therapeutic regimens employed are highly effective, the number of expected relapses was low, necessitating caution when evaluating the results. Nevertheless, it is important to note that four of the patients were included in the study due to relapse of brucellosis which had been treated elsewhere, and all of them had a positive PCR at the time of admission. This agrees with previous results reported by our group in another study (21). Thus, if we include the two episodes of relapse in this study, we have had the opportunity to perform PCR analysis for 14 episodes of relapse so far. Of these, the blood cultures were positive in 10 (71.4%) episodes compared to the 13 (92.8%) positive PCR results (15, 21).

As well as correctly identifying the two relapsed patients, the PCR was negative in all patients in whom a suspected relapse was ruled out. This seems to provide the test with a high negative predictive value, a very important fact if we consider that in two of these four patients with unconfirmed suspected relapse, the titers of the Coombs’ test had increased significantly. Similar serological findings have been reported by others (4, 13, 20). Finally, only 1 of the 77 PCRs performed between the second and sixth months of follow-up in the patients who had no relapse was false positive (1.3%). In this case, the patient had returned to work with his cattle, suggesting that he may have had a subclinical reinfection not detected by the other microbiological techniques.

Finally, although a few patients may have a positive PCR at conclusion of treatment, and it is necessary to be prudent in interpreting the results in patients who are permanently exposed, PCR appears to be a more sensitive technique than conventional microbiological methods, not just for the diagnosis of a first episode of infection, but also for posttherapy follow-up of the disease and the early detection of relapses.

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