PCR Assay for Diagnosis of Human Brucellosis

We have read with interest the recent article by Morata et al. reporting their study of two of the possible factors which can interfere with specific DNA amplification in a peripheral-blood PCR assay used for the diagnosis of human brucellosis (2). The study deals with the optimization of a method described in a previous work by these authors (4), in which they report a sensitivity of 100% and a specificity of 98% for rapid diagnosis of human brucellosis by PCR. Taking into account the clinical importance of this finding, we have tried to reproduce these results with 10 patients diagnosed with acute brucellosis and 5 healthy subjects. The diagnostic criteria were the isolation of a Brucella sp. from blood (n = 6) or the finding of a ≥1/160 standard tube agglutination titer of antibodies to Brucella, in association with compatible clinical findings (n = 4). Isolation of DNA from blood samples and DNA amplification were carried out by following the technique described by Morata et al. (2). All PCRs were carried out in duplicate.

The results are shown in Table 1. The sensitivity was 50% (confidence interval [CI], 95%; 18.7 to 81.3%), and the specificity was 60% (CI, 95%; 14.7 to 94.7%). Curiously, of the samples from the six patients with brucellosis from which Brucella melitensis was isolated by blood culture, four were PCR negative. The existence of false negatives for patients with positive blood cultures could be due to the presence of polymerase inhibitors. Many substances have been suggested to be amplification inhibitors, including hemoglobin, urine, heparin, phenol, and sodium dodecyl sulfate. The technique described by Morata et al. seems to overcome inhibition caused by some of them, such as hemoglobin and heme compound derivatives. Nevertheless, we have not been able to reproduce the results of these authors, although we carefully followed the exact indications that are published in their article. Other hypotheses to account for those false-negative PCR results are the presence of a number of organisms below the detection limit and the degradation of target DNA in the samples (5).

False positives for healthy subjects could be due to (i) asymptomatic infections not detectable by conventional diagnosis procedures (blood cultures and serology) or (ii) cross-reactions with DNA from other bacteria, as described by Romero et al. (5). Those authors have developed a PCR assay with primers F4 and F5, derived from the 16S rRNA sequence of Brucella abortus. Romero et al. carried out a very detailed specificity study, and only Ochrobactrum anthrofi biotype D yielded a PCR product of the expected size, suggesting a close relationship between Brucella spp. and O. anthrofi biotype D. When applying these methods to human samples (6), these authors found problems with false positives. The presence of O. anthrofi DNA in human blood could be detected by this method. Even though the natural habitat of this bacterium is unknown, it has been isolated from different kinds of samples, among these, clinical samples from immunodepressed patients as well as from healthy individuals (6). Baily et al. (1) did not test whether the B4-B5 primers do amplify the DNA of O. anthrofi. If this hypothesis is true, it could explain our false positive. Lastly, the differences between our results and those of Morata et al. could be due to the difficulty in reproducing PCR results in distinct laboratories, as described by Noordhoek et al. (3).

<p>| TABLE 1. Results of PCRs with samples from patients with acute brucellosis and from healthy subjects |
|---------------------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Patients with acute brucellosis</th>
<th>Healthy subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR positive</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>PCR negative</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>5</td>
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REFERENCES

Authors’ Reply
We thank Navarro et al. for their interest in our work and regret their difficulties in being able to reproduce results similar to those of our group. In fact, their results with a small group of patients and controls are rather surprising, as are some of the comments they make to justify them. In theory at least, it is paradoxical that the PCR false negatives should occur in precisely those patients with a positive blood culture, and we do not think it reasonable to attribute this to a bacterial inoculum below the PCR detection threshold. Zimmerman et al. demonstrated the existence of a close relationship between the size of the inoculum and the positivity of the blood culture and growth rate of the organism (8). The results obtained by
Navarro and colleagues would, in fact, point in just the opposite direction to these findings. In one of our works we reported that the detection level of the technique was around 10 fg of DNA, equivalent to the DNA from two cells (5). This amount of inoculum is so small that it is difficult to believe that it would not exist in 0.5 to 1 ml of blood from most patients with positive blood cultures. Moreover, since our first report we have studied a further 52 cases of brucellosis by means of PCR, 36 (69.2%) patients with positive blood cultures and 16 (30.8%) diagnosed according to conventional clinical and serological criteria. Overall, 48 of these 52 patients (92.3%) had a positive PCR, corresponding to 34 (94.4%) of those with a positive blood culture and 14 (87.5%) of those diagnosed clinically and serologically. Although we are unaware of the conditions of extraction and storage for the samples described by Navarro et al., degradation of the DNA sample seems an unlikely reason for the high rate of false PCR negatives since we obtained satisfactory results from samples maintained at −20°C for 6 months prior to processing.

The area where our center is situated and that where Navarro et al. work are both regions where brucellosis is endemic. Virtually 100% of reported cases of brucellosis in Spain are caused by *B. melitensis*, which is recognized as the most virulent biovar of the *Brucella* genus. Gotuzzo et al. reported that the rate of clinical infection with *B. melitensis* in an exposed population was higher than 50% (4). The existence of asymptomatic brucellosis is well-known, but this does not appear to be very common in the case of *B. melitensis*, and, at the present time, in order to speak strictly of an asymptomatic infection it is necessary to isolate the causative agent or demonstrate some type of specific serological response. Since we included in all our PCRs a sample from a healthy subject as a control of the process of DNA extraction, and to date we have had no false-positive results due to this, we do not think that the existence of an asymptomatic infection is the cause of false positives; nor, therefore, does it contribute significantly to a reduction in the specificity of the technique.

The close phylogenetic relationship between *O. anthropi* and *Brucella* spp. is acknowledged, as is the observation of similar products amplified by using the 31-kDa *Brucella* protein, the heat shock proteins (DnaK, DnaJ, HtrA and GroEL), and 16S rRNA primers (1). Nevertheless, we agree with Romero et al. that “it is unlikely that *O. anthropi* would cause a false-positive result in a test for *Brucella* spp. with the PCR assay . . . since *O. anthropi* has rarely been found to be pathogenic” (6). Given the low virulence of this microorganism, it would be surprising if it infected healthy persons and produced asymptomatic bacteremia leading to a false-positive PCR result. With respect to this, it is interesting that only a very few cases of infection by *O. anthropi* have been reported to date and that almost all occurred in severely immunosuppressed patients or those with debilitating illnesses; most infections were nosocomial or in patients with catheters or other foreign bodies (2, 3). From a clinical point of view, this is a situation diametrically opposite to infection with *Brucella* spp., which is always a community infection affecting generally immunocompetent subjects.

Finally, we agree with Navarro et al. that in-house PCR results can sometimes be difficult to reproduce. PCR, although a theoretically simple concept, requires dedicated and experienced personnel. The adaptation and acceptance of this technology in the forum of clinical diagnosis has been slow, due mainly to a number of technical obstacles (7). We are sure that familiarization with the technique will eventually lead to these authors producing results similar to those communicated by our group.

**REFERENCES**


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