Am. J. Trop. Med. Hyg., 76(4), 2007, pp. 698–702

EVALUATION OF BRUCELLOSIS BY PCR AND PERSISTENCE AFTER TREATMENT IN PATIENTS RETURNING TO THE HOSPITAL FOR FOLLOW-UP

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Abstract. Polymerase chain reaction (PCR) was applied to confirm the diagnosis of brucellosis and to study its clearance in response to the standard treatment regimen with doxycycline and rifampin at hospitals in Callao and Lima, Peru. The PCR confirmed the diagnosis in 23 (91.7%) patients with brucellosis including 12 culture-confirmed cases. For patients treated at the hospital in Callao, PCR was positive for all samples collected during and at the conclusion of treatment and for 76.9% of follow-up samples collected on average 15.9 weeks after completion of treatment. For patients treated at the hospital in Lima, PCR tests were positive for 81.8% of samples collected during treatment, for 33.3% of samples collected at the conclusion of treatment, and for ≥ 50% of samples collected at first, second, and third post-treatment follow-up. Thus, *Brucella* DNA may persist in the serum weeks to months after completion of the standard treatment regimen.

INTRODUCTION

Brucellosis is a worldwide zoonosis[1] that affects both farm animals and humans.[2–4] In Peru, > 1,000 cases of human brucellosis are reported annually, with ~27% of the cases being reported in Callao, the harbor city of Lima that accounts for 3.4% of the Peruvian population.[5] Earlier studies have shown that most cases are caused by *B. melitensis,* which is consistent with the presence of brucellosis in goats at farms in provinces neighboring Lima, from where dairy is imported into the city. In Peru, dishes prepared with soft cheese are popular and potentially present a rich source of infection.

Brucellosis is an intracellular pathogen in cells of the immune system and therefore difficult to diagnose and treat.[6] The clinical presentation is non-specific and requires laboratory testing for confirmation.[2] Treatment failure and relapse rates can be high and depend on the drug combination and compliance.[7–10] Culture provides direct evidence of the presence of the pathogen and is the gold standard,[11] but in the absence of adequate culture facilities, brucellosis might be diagnosed by serologic testing.[12] Recently, polymerase chain reaction (PCR) was applied to follow-up samples from patients with brucellosis treated with either doxycycline alone or with doxycycline plus gentamycin. It was shown that the bacterial DNA persists in the blood of several patients throughout treatment and follow-up despite apparent clinical recovery.[13]

In this study performed at two hospitals in Peru, we applied the BCSP31-PCR assay with the genus-specific B4 and B5 primers for the amplification of a 223-bp segment of the sequence encoding a 31-kd *B. abortus* protein[14] to confirm the diagnosis and to assess the persistence of pathogen DNA in the serum of patients treated for brucellosis with doxycycline and rifampin.

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MATERIALS AND METHODS

We retrospectively examined two groups of serum samples collected at two different hospitals in Peru. The first group ($N = 74$) was collected at the Hospital Nacional Daniel Alcides Carrión (HNDAC) in Callao, between January and October 2005; the second group ($N = 77$) was collected at Hospital Nacional Arzobispo Loayza (HNAL) in Lima between December 1999 and January 2001. The first group was selected based on volume and availability of serologic data in the laboratory record forms and can be further divided into the following three subgroups: 1) 13 Rose-Bengal (RB)-positive samples that had been collected at the time of first diagnosis of brucellosis, 2) 22 RB-positive and 1 RB-negative follow-up sample collected either during, at the conclusion of, or after the completion of treatment from 17 patients with brucellosis, and 3) 38 RB-negative initial samples collected from patients with clinical suspicion of brucellosis and a final diagnosis other than brucellosis. From five of the patients diagnosed with brucellosis and a sample collected at first diagnosis, either one or two follow-up samples were also available for testing. From the other patients, either only an initial sample or one or more follow-up samples were present. The samples from HNAL were collected at first diagnosis and during follow-up ($N = 51$) from 13 patients with brucellosis and at first diagnosis from 13 patients without brucellosis. Three to six follow-up samples collected during treatment, at the conclusion of treatment, and up to > 1 year after the end of treatment were available from each patient with brucellosis. All samples had been stored at –20°C until use.

At HNDAC, the tube agglutination test (TAT) was used for the confirmation of brucellosis and the 2-mercaptoethanol (ME) test was used to monitor disease activity and response to treatment. Blood cultures in addition to serologic testing with the plate agglutination test (PAT) were used for the confirmation of brucellosis at HNAL. Patients diagnosed with brucellosis were treated with doxycycline and rifampin according to the World Health Organization (WHO) regimen. The RB, TAT, PAT, and 2-ME tests were performed using antigens obtained from the Peruvian National Institutes of

Health.[12,15] Results for the agglutination tests were obtained from laboratory records. Results of the TAT and PAT were considered consistent with brucellosis for titers ≥ 1:200. The 2-ME test was considered positive for titers ≥ 1:100, and a 1-fold titer reduction or more was considered significant when monitoring the response to treatment. Result of the TAT was not recorded for one RB-positive sample collected at first diagnosis.

The *Brucella* IgM/IgG immunochromatographic lateral flow assay was applied for the detection of *Brucella*-specific IgM and IgG antibodies in the serum samples.[16] The flow assay was performed as described previously, and positive test results were subjectively rated 1+ when weak, 2+ when moderately strong, 3+ when strong, and 4+ when very strong.[16]

For PCR analysis, DNA was extracted from 300 μL serum using the QIAamp (Qiagen, Valencia, CA) DNA blood mini kit according to the manufacturer's instructions and was eluted in a 50-μL eluent supplied with the kit. DNA amplification using the B4/B5 primer pair originally described by Baily and others[14] was performed on 7.5 μL of the DNA sample in a 37.5-μL reaction mixture containing 10 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 0.5 μmol/L of each of the two primers, and 0.94 U of Taq polymerase (Invitrogen, Carlsbad, CA). After an initial denaturation step at 93°C for 5 minutes, 40 amplification cycles were performed, each consisting of 1 minute at 90°C, 30 seconds at 60°C, and 1 minute at 72°C, followed by a final extension step at 72°C for 7 minutes. An experiment in which 300 μL serum was spiked with *Brucella* DNA indicated an analytical sensitivity of one genomic copy per microliter of serum using the above extraction and amplification protocol. All PCRs were performed in duplicate with the appropriate inclusion of negative and positive controls. A PTC-100 programmable thermal controller from MJ Research (Waltham, MA) was used to carry out the PCR program. The volumes of one RB-positive sample collected at the time of initial diagnosis at HNDAC and of four final follow-up samples from HNAL were not sufficient for DNA extraction. Strict precautions including working in separate rooms for master mix preparation, sample extraction, and PCR analysis were taken to prevent contamination. In addition, different laboratory staff performed the DNA extractions and PCR reactions.

Approval from the Medical Ethics Committees of HNDAC, HNAL, and A.B. PRISMA were obtained before the start of the study.

RESULTS

The diagnosis of brucellosis was confirmed by serologic testing in the *Brucella* IgM/IgG flow assay for the samples from HNDAC and by blood culture and serologic testing in the flow assay for the samples from HNAL. For the samples from HNDAC, the flow assay tested positive for all RB-positive initial samples and negative for all RB-negative initial samples, with 100% of the RB-positive samples giving a ≥ 2+ staining intensity for IgG and 81.8% of them giving a ≥ 2+ staining intensity for IgM. For the samples from HNAL, culture was recorded to be positive for 12 of the 13 patients with brucellosis, and the flow assay tested positive for all initial samples from these patients and negative for all RB-negative control samples, with all but one of the flow assay–positive samples giving a ≥ 2+ staining intensity for IgM, IgG, or both.

The BSCP31-PCR gave a positive result for all 12 (100%) tested RB-positive initial serum samples from patients diagnosed with brucellosis at HNDAC and tested positive in 20 (87.0%) of the 23 follow-up samples (Table 1). The median titers in the TAT and the 2-ME test for the group of initial samples were 1:400 (range, 1:100–1:1,600) and 1:100 (range, 1:25–1:400), and for the group of follow-up samples, these values were 1:100 (range, 1:25–1:400) and 1:25 (range, no agglutination to 1:400), respectively. Of the control group, three (7.9%) samples tested positive by PCR, and one of these PCR-positive controls agglutinated in the TAT with a titer of 1:200.

Of the 20 PCR-positive follow-up samples from HNDAC, 2 had been collected during treatment, 4 at the conclusion of treatment, and 12 samples from 10 patients were collected up to 27 weeks (mean, 15.9 weeks; range, 9–27 weeks) after treatment ended. For two samples, it was not possible to determine collection dates. Two of the three PCR-negative follow-up samples were collected > 2 years after first diagnosis. The duration of follow-up for the third negative sample was not recorded.

To confirm the observation that *Brucella* DNA may persist in the serum of treated patients, we studied initial and follow-up samples from 13 patients who presented with brucellosis at HNAL. The PCR tested positive in 11 (84.6%) initial samples including the sample from the culture-negative patient (Table 2). For one PCR-positive patient, only follow-up samples collected during and at the end of treatment were available, and these all tested positive. In two PCR-positive patients, the PCR was positive for the samples collected during and at the end of treatment and negative for all samples collected after the end of treatment. These PCR-negative samples were collected at the 2nd and 4th month of follow-up in one patient and during the 3rd, 4th, and 12th month in the other patient. A culture-positive patient that tested negative by PCR in the initial sample relapsed 6 months after the initial diagnosis. The relapse was confirmed by culture. A serum sample taken 130 days before the relapse was also negative by PCR. However, the sample collected at the time of the relapse and a sample collected again 3 months later tested PCR positive. In the other nine patients, the PCR was either positive for all follow-up samples collected during, at the conclusion, and after the completion of treatment or intermittently positive and negative. The PCR-positive post-treatment follow-up

TABLE 1

Results of different serologic tests and the BCSP31-PCR for RB-positive and -negative serum samples collected at HNDAC* from patients with clinical suspicion of brucellosis during their initial evaluation and follow-up

Patients and serum group	No. positive in the following assays/no. of samples (percentage positive)		
	TAT	2-ME	PCR
Patients with brucellosis			
Initial (N = 13)	11/12 (91.7)†	7/13 (53.8)	12/12 (100)‡
Follow-up (N = 23)	9/23 (39.1)	3/23 (13)	20/23 (87)
Patients with illness other than brucellosis			
Initial (N = 38)	1/38 (2.6)	0/38 (0)	3/38 (7.9)§

* HNDAC, Hospital Nacional Daniel Alcides Carrión.
† Results of TAT was not recorded for one sample.
‡ The volume of one sample was too small to perform PCR.
§ PCR was performed on 31 samples.

TABLE 2

Results of the PAT and the BCSP31-PCR for samples collected at different stages of diagnosis, treatment, and follow-up from patients with brucellosis treated at HNAL*

Group and period of collection in weeks after diagnosis (no. of samples)	No. positive in the following assays/no. tested (percentage positive)		Average sampling time of PCR-positive samples in weeks after diagnosis (range)
	PAT	PCR	
Patients with brucellosis			
Initial ($N = 13$)	11/13 (84.6)	11/13 (84.6)	
Follow-up			
During treatment† 1.4–5 ($N = 11$)	9/11 (81.8)	9/11 (81.8)	3.1 (1.4–5)
At conclusion of treatment 5.1–7.9 ($N = 9$)	5/9 (55.5)	3/9 (33.3)	6.3 (5.6–6.9)
After the end of treatment‡			
8.6–32.4 ($N = 13$)§	9/13 (69.2)	7/12 (58.3)¶	18 (8.6–32.4)
11.3–36.6 ($N = 11$)	5/11 (45.5)	5/10 (50)¶	24.9 (12.9–36.6)
19.3–59 ($N = 4$)	1/4 (25)	2/3 (66.7)¶	22.6 (19.3–25.7)
Patients with an illness other than brucellosis			
Initial ($N = 13$)	0/13 (0)	1/13 (7.8)	

* HNDAC, Hospital Nacional Daniel Alcides Carrón.
† Two samples collected 2 days after the initial samples are not included.
‡ Samples were grouped in first, second, and third samples collected after the conclusion of treatment.
§ One sample collected 2 days after the conclusion of treatment is not included.
¶ There was not enough volume to perform PCR on one sample of this group.

samples were collected up to 53 weeks (mean, 15.9 weeks; range, 2.5–53 weeks) after treatment ended. PCR positivity was 81.8% for samples collected during treatment, 33.3% for samples collected at the conclusion of treatment, and was $\geq 50\%$ for the first, second, and third post-treatment follow-up samples collected on average 16.2, 22.2, and 37.2 weeks after the end of treatment, respectively (Table 2). The PCR-positive post-treatment first, second, and third follow-up samples were collected on average 18 (range, 2.6–26.4), 18.9 (range, 6.9–30.6), and 16.6 (range, 13.3–19.7) weeks after the end of treatment. In seven patients with post-treatment follow-up, the final post-treatment follow-up sample was PCR positive, and these samples were collected on average 15.7 weeks (range, 6.9–26.3 weeks) after the end of treatment. In the three other patients with post-treatment follow-up, the final samples were PCR negative, and in these patients, the PCR-positive samples were collected 4.7, 8, and 26.1 weeks after the end of treatment. All patients were asymptomatic at the end of the follow-up period. PCR tested positive for the initial sample from 1 (7.9%) of the 13 control patients.

χ^2 for trend analysis showed a decrease in the percentage of serum samples that tested PCR positive at first diagnosis and during the successive stage of follow-up (Table 3; $P = 0.005$). However, no decrease was observed ($P = 0.24$) if considering the percentage of patients with a PCR-positive result for one or more samples collected during post-treatment follow-up. This result may indicate that, while the pathogen persists in the serum of treated patients, the absolute amount decreases, thereby reducing the chance that the DNA is detected in the PCR.

TABLE 3

Persistence of *Brucella* DNA in patients treated for brucellosis at two hospitals in Peru and reduction in the number of PCR-positive samples at the successive stage of treatment and follow-up

Stage and statistical analysis	Percentage of PCR-positive samples (95% CI)	Percentage of PCR-positive patients (95% CI)
Initial	92.0 (81–100)	92.0 (81–100)
During treatment	84.6 (64–100)	84.6 (64–100)
End of treatment	53.8 (25–82)	53.8 (25–82)
Post-treatment follow-up	62.5 (39–88)	84.0 (69–99)
χ^2 for trend	$P = 0.005$	$P = 0.24$

DISCUSSION

An immunochromatographic lateral flow assay and a PCR were applied to confirm the diagnosis in patients treated for brucellosis at the hospital in Callao because many of these patients return to the hospital because of complaints suggesting either misdiagnosis or poor response to treatment. The flow assay and the PCR confirmed the diagnosis in all patients. Furthermore, it was noted that the majority of the follow-up samples tested PCR positive, which suggests a poor response to treatment. This observation was confirmed by testing a second group of samples collected at first diagnosis, during and at the end of treatment, and during post-treatment follow-up from culture-confirmed patients with brucellosis treated at a hospital in Lima. The results suggested that the DNA of the pathogen and perhaps *Brucella* itself persists for weeks to months after the conclusion of treatment in the serum from patients treated for brucellosis and who return to the hospital with complaints. The possibility of poor compliance cannot be excluded, and the results should be confirmed by culture.

Combined for the two hospitals, the BCSP31-PCR tested positive in 91.7% of the initial samples collected from patients diagnosed with brucellosis. Previous studies have shown that PCR has a high sensitivity and specificity and only few false-positive results have been reported, all for samples from patients with tuberculous vertebral osteomyelitis.[17–22] In this study, PCR tested positive in four patients, three at HNDAC and one at HNAL, who had a final diagnosis other than brucellosis, and the sample of one of these patients also agglutinated in TAT at a titer of 1:200. Therefore, a false-positive result should be considered for at least the three patients with a negative serology. No false-positive results were observed in the flow assay, and this is consistent with the high sensitivity and specificity of this test.[16,23]

Serum samples collected after the end of treatment from 10 (76.9%) patients treated at HNDAC and from 11 (84.6%) patients treated at HNAL tested positive with the PCR, in-

dicating the persistence of pathogen DNA in serum. Patients were treated with a standard regimen of doxycycline and rifampin, and all patients were considered cured at the end of follow-up. PCR-positive post-treatment samples were collected on average ~4 months after the end of treatment. One patient had a culture-confirmed relapse and tested positive with PCR at the time of relapse. This confirms and extends the observation made by Navarro and others,[13] showing that 50% of the patients had not cleared the pathogen as determined by PCR after finalizing treatment and 40% had not done so at the end of follow-up 1–2 years after the end of treatment.[13] Notably, seven patients in that study relapsed. A quantitative real-time PCR system was used, and no difference in the evolution of the DNA load was observed between patients who relapsed and those who did not. Some of the patients followed in this study were intermittently positive, suggesting that the analytical sensitivity of the BCSP31-PCR may limit the detection of the pathogen in serum when very low DNA levels are present.

In an earlier study, positive PCR results during follow-up were observed for samples collected from two relapsing patients; in another case, the positive result was thought to be caused by a subclinical infection after occupational exposure.[24] Although the same PCR system was used as in our study, we used a different DNA extraction procedure and performed five more amplification cycles compared with the original protocol. This may well have resulted in a better analytical sensitivity of the PCR and hence may explain the detection of *Brucella* DNA in the follow-up samples in a higher percentage of patients. In a Saudi Arabian study, follow-up samples collected after the end of treatment from 2 of 20 patients tested PCR positive[25]; importantly, the presence of *Brucella* in the blood of these patients was confirmed by culture.

The 2-ME test is sometimes used to monitor response to treatment.[26] The low 2-ME titers in the follow-up samples seem to contrast with the persistence of the pathogen DNA in treated patients.

Earlier studies have indicated that the existing WHO treatment regimens for brucellosis are not completely successfully in treating patients and avoiding relapses.[22,9,10,27,28] In Peru, most of the patients who return for follow-up have complaints, and additional studies will be needed to determine the percentage of treated patients in whom the DNA of the pathogen persist. The detection of pathogen DNA in the serum of treated patients who are still symptomatic may indicate the necessity for further treatment. The management of asymptomatic patients who test positive in PCR still requires some caution. Further studies including larger numbers of patients and using cultures are needed to confirm our observations and to determine whether persistence of *Brucella* DNA in serum reflects the presence of active bacteria and whether this leads to recurrent disease at a later stage. Confirmation of our findings by culture and demonstration of a correlation with the occurrence of complications or clinical outcomes may support the clinical relevance of the detection of the DNA in the serum.

Received August 29, 2006. Accepted for publication November 1, 2006.

Acknowledgments: The authors appreciate the useful advice provided by Dr. P. Moro. The other members of the Brucellosis Working Group in Callao members are L. Castañeda-Castañeda, M. Mendoza-Núñez, E. Yagui, P. Tuesta (Hospital Nacional Daniel Alcides Carrión, Callao, Peru), and T. H. Abdoel (Royal Tropical Institute, Amsterdam). The authors thank Dr Stella van Beers for statistical analysis and Yvonne Ahn for assistance in editing this manuscript.

Financial support: M. Méndez, M. Mulder, and M. P. Franco were supported by the Infectious Disease Training Program in Peru (5 D43 TW006581-04), and H. L. Smits received support from EU Cost Action 845.

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