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Chronic Brucellosis and Persistence of *Brucella melitensis* DNA▽

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After acute brucellosis infection, symptoms persist in a minority of patients for more than 1 year. Such patients are defined as having chronic brucellosis. Since no objective laboratory methods exist to confirm the presence of chronic disease, these patients suffer delays in both diagnosis and treatment. The aim of the current study was to evaluate the usefulness of quantitative real-time PCR (Q-PCR) in the diagnosis and follow-up of these patients. Thirty-five subjects with a well-documented history of brucellosis that had been diagnosed between 2 and 33 years previously were screened by Q-PCR for the presence of *Brucella melitensis* DNA and by serological tests and blood culture. Subjects were divided into three groups: 8 (23%) focal-disease subjects, 9 (26%) nonfocal-disease subjects with subjective complaints, such as fatigue, malaise, arthralgia, and/or myalgia, and 18 (51%) asymptomatic subjects. All (100%) focal-disease patients and symptomatic nonfocal-disease patients had at least one positive Q-PCR sample. Only six (33%) of the asymptomatic subjects had Q-PCR-positive samples ($P < 0.05$). Eleven patients (five focal-disease patients and six nonfocal-disease patients with subjective complaints) received therapy during the study. For those patients who completed treatment, six (60%) still had Q-PCR-positive samples at the posttreatment follow-up. The proportion of individuals with *B. melitensis* DNA was significantly higher for symptomatic nonfocal-disease patients than for asymptomatic subjects. Therefore, Q-PCR appears to be a useful method for identifying chronic brucellosis patients.

Human brucellosis is a multisystem disease that may present with a broad spectrum of clinical manifestations. The causative organisms, *Brucella* spp., are facultatively intracellular bacteria that are capable of evading a number of host defense mechanisms and can survive within phagocytic cells for long periods. These properties may account for focal complications, relapses, and chronic disease (21).

The chronic course of the disease was initially explored during the 1930s by Evans (6a) and was further explored in the 1950s by Davies (5). In 1951, Spink and associates stated, with respect to the duration of the illness, that the majority of patients with brucellosis recover within a year after the administration of an antibiotic, while a small but significant number of patients continue to have clinical manifestations despite such therapy (22). These patients can be divided into two groups: those with a focal disease, such as spondylitis, and those without a focal disease who complain, nevertheless, of poor health and have symptoms such as chronic fatigue syndrome (CFS), musculoskeletal pain, depression, or anxiety.

The diagnosis of chronic brucellosis is often based on clinical complaints together with the presence of high immunoglobulin G titers (2). However, the specificity of current serological assays is considered to be low, since titers may remain positive for years after the successful resolution of symptoms. In 1980, Buchanan and Faber described a serological test for evaluation of the effectiveness of treatment and for excluding a diagnosis of chronic brucellosis (3). The authors observed that, in assays with 2-mercapthoethanol, results remained positive for 9% of patients 1 year after the initiation of treatment. Among these patients, 50% still had signs and symptoms of brucellosis and required further treatment. Recently, PCR has been used to detect *Brucella* spp. in the diagnosis of primary infections, relapse, and focal complications of the disease (12, 13, 20). Initially, the persistence of *Brucella* sp. DNA after therapy was linked to relapse (9, 12, 17). However, our group and others have demonstrated the persistence of *Brucella* sp. DNA for long periods of time after the conclusion of therapy in asymptomatic patients (10, 15, 24). The practical role of quantitative real-time PCR (Q-PCR) in the laboratory diagnosis of chronic brucellosis and the assessment of clinical manifestations remains to be demonstrated. In the present study, we examined 35 subjects with a well-documented history of brucellosis and followed them to evaluate the usefulness of Q-PCR and the relationship between a positive result by the Q-PCR assay and clinical course.

MATERIALS AND METHODS

Subjects and samples. Between February 2003 and October 2008, we screened patients with a well-documented history of brucellosis for symptoms and the persistence of *Brucella melitensis* DNA. A diagnosis of acute brucellosis had been made between 2 and 33 years previously according to one or both of the following criteria: isolation of *Brucella* spp. from blood or any sample of body fluid or tissue and/or the presence of a compatible clinical picture together with the demonstration of specific antibodies at significant titers (≥1:160 by the Wright test or ≥1:320 by the Coombs test) or seroconversion. All of the isolated strains were sent to the Centro de Investigación y Tecnología Agroalimentaria (Zaragoza, Spain) for definitive identification and biotyping. All isolates were identified as *B. melitensis* biotype 1 and *B. melitensis* biotype 3.

Subjects were divided into three groups. Group A consisted of eight (23%) focal-disease subjects. Group B comprised nine (26%) nonfocal-disease subjects who complained of subjective symptoms, such as fatigue, malaise, arthralgia, and/or myalgia. Group C included 18 (51%) asymptomatic subjects.

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Each time a patient visited our office for a checkup, blood and serum samples were taken for analysis by Q-PCR, blood culture, and serological and laboratory evaluations. Laboratory evaluations included complete blood counts, erythrocyte sedimentation rates, routine biochemical tests (for alanine aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase, and creatinine), and tests for C-reactive protein and rheumatoid factor. We also analyzed one sample of cerebrospinal fluid (CSF) and one sample of synovial fluid (SF) for two focal-disease patients.

Definitions. Because we observed various patterns of disease, we used the following operational definitions for purposes of clarity and classification. Chronic brucellosis patients included all patients whose symptoms, whether nonspecific or symptoms of focal disease, had persisted for more than 1 year after the initial episode. Diagnosis of focal disease, such as spondylitis, sacroiliitis, hip arthritis, knee arthritis, spleen abscess, hepatic abscess, or multifocal motor neuropathy, was made on the basis of appropriate findings upon physical examination and radiological analysis, bone scintigraphy, magnetic resonance studies, or electroneurograms.

Treatment. Treatment regimens were based on patients' preferences and/or focal complications. Therapeutic efficacy was evaluated as either (i) a treatment effect of no benefit or a (ii) temporary or (iii) permanent resolution of symptoms or signs of the disease at the end of antibiotic treatment.

Control group. Control samples of whole blood and serum were obtained from 15 healthy donors at the University General Hospital of Albacete (Albacete, Spain). The study was approved by the institutional review board at the center, and informed consent was obtained from all patients.

Microbiological studies. Tissue samples were cultured according to standard microbiological techniques (7). Blood culture, the rose bengal test, and the Wright test were performed as described elsewhere (15). The anti-*Brucella* Coombs test was performed by the standard method (19).

Extraction of DNA. DNA was extracted from whole-blood samples, serum samples, CSF, and SF with the UltraClean DNA-BloodSpin kit (Mo Bio Laboratories, Carlsbad, CA) in accordance with the manufacturer's instructions but with the following three modifications: (i) in step 1, 40 μl of proteinase K (6 mg/ml) was added; (ii) in step 6, incubation at 65°C was performed for 15 min; and (iii) in the final step, DNA was eluted in 50 μl of elution buffer for samples of whole blood, CSF, and SF and in 40 μl for serum samples. All of the final concentrations of DNA, as well as purity, were determined with a Nanodrop spectrophotometer, model ND-1000 (Nanodrop Technologies, Wilmington, DE).

Q-PCR. After DNA extraction, amplification was performed as previously described (15) with slight modifications. The amount of human DNA included in each 20 μl of Q-PCR mixture was 200 ng when DNA had been extracted from whole-blood samples and 2 μl when DNA had been extracted from serum, CSF, or SF. For construction of a standard curve, 10-fold serial dilutions of the 251-bp fragment of *B. melitensis* DNA were included in duplicate, at levels ranging from 10^6 to 10^0 copies per reaction mixture. The standard curve had a correlation coefficient (R^2) of 1, a slope of −3.4, and an intercept of 40. Q-PCR efficiency ($E = 10^{-1/\text{slope}}$) was 1.97.

To avoid potential carryover contamination of samples with previously amplified products of Q-PCR, tubes with water only were distributed among the tubes with clinical samples, and all were handled in the same way as the tubes of samples during DNA extraction and Q-PCR. The mixture for Q-PCR was prepared in a laminar-flow hood, and positive controls were manipulated in a separate room with a different set of instruments.

Each sample was analyzed in triplicate. A sample was considered positive when at least one of the three replicates gave an amplified product. Each assay included one positive control consisting of a dilute solution of *B. melitensis* DNA and one negative control lacking template DNA. Data (bacterial DNA loads) were expressed as copies of *B. melitensis* DNA per milliliter of sample ± standard deviation (SD).

Statistical analysis. The chi-square test and Fisher's exact test were used to compare categorical variables in different groups. Student's *t* test and the Wilcoxon rank-sum test were used to compare continuous variables. A *P* value equal to or less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

Study subjects. There were 35 subjects in the study, each with a well-documented history of brucellosis, diagnosed between 2 and 33 years previously. Twelve subjects were women (34%), and 23 were men (66%). The mean age was 48 years (range, 24 to 83 years). Subjects were followed for 1 to 2,166 days (mean, 657 days). Patients' epidemiological features and clinical findings upon entry into the study are summarized in Table 1. Diffuse polyarthralgia and fatigue were the most frequent symptoms. None of the patients was febrile. Five patients had been diagnosed with CFS and one with fibromyalgia.

Serological analysis and blood culture. We performed serological analysis on a total of 246 serum samples, including 90 from focal-disease patients, 122 from symptomatic nonfocal-disease patients, and 34 from asymptomatic subjects. The number of serologically positive samples tested for each group of patients is summarized in Table 2.

None of the 60 blood samples collected yielded positive cultures. The sample of CSF collected from the patient with neuropathy and the sample of SF from the patient with chronic right-knee pain also gave negative results in culture.

Quantification of *B. melitensis* DNA loads in clinical samples. The numbers of positive Q-PCR samples and the quantification of *B. melitensis* DNA loads over time for the three groups of subjects are summarized in Table 3.

All (100%) symptomatic focal-disease patients and nonfocal-disease patients with subjective complaints had at least one Q-PCR-positive sample (whole blood and/or serum). This percentage was significantly higher than the 33% (6 out of 18 patients) of asymptomatic subjects who tested positive by Q-PCR. Concerning bacterial load, statistically significant differences were found between groups A and B, B and C, and C and A in both whole-blood and serum samples collected at baseline.

Group A: focal-disease patients. Three to 28 serial whole-blood and serum samples were obtained from each focal-disease patient. In total, 190 samples, including 95 whole-blood samples, 93 serum samples, 1 CSF sample, and 1 SF sample, were analyzed by Q-PCR. At baseline, the mean bacterial loads were 244 ± 253 copies/ml (12/39 samples positive) and 432 ± 505 copies/ml (7/38 samples positive) for the whole-blood and serum samples, respectively ($P < 0.05$). The SF sample yielded a positive result, with 175 copies of *B. melitensis* DNA per ml. The CSF sample was negative.

Group B: nonfocal-disease subjects. Nine to 21 serial whole-blood and serum samples from each symptomatic nonfocal-disease patient were analyzed by Q-PCR. In total, 234 samples, including 118 whole-blood and 116 serum samples, were included. At baseline, the mean bacterial DNA loads were 515 ± 1,524 copies/ml (14/67 samples positive) and 243 ± 222 copies/ml (12/65 samples positive) for the whole-blood and serum samples, respectively ($P > 0.05$).

Group C: asymptomatic subjects. One to 12 serial whole-blood and serum samples were obtained from each asymptomatic subject, with 36 whole-blood and 36 serum samples in total. The bacterial DNA loads for these six subjects were 171 ± 136 copies/ml (5/36 samples positive) and 53 ± 11 copies/ml (2/36 samples positive) for whole-blood and serum samples, respectively ($P < 0.05$).

Control subjects. All of the 15 whole-blood and 15 serum samples from the 15 healthy donors that were analyzed by Q-PCR produced negative results.

***B. melitensis* DNA changes in treated chronic brucellosis patients.** Eleven patients (five focal-disease patients and six symptomatic nonfocal-disease patients) received one to four courses of antibiotics, consisting of doxycycline (100 mg/12 h)

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TABLE 1. Clinical and epidemiological details and results of serological tests and Q-PCR assays of 17 chronic brucellosis patients upon entry into the study

Patient no.	Dates of sample collection	Age (yr)/ sex[a]	Yr of infection	Focal complication	Associated disease[b]	Clinical manifestation(s)	Result by the following test[c]:					Treatment (days)[d]	Outcome
							RB	STA	Coombs	Q-PCR			
										Blood	Serum		
Group A (focal-disease patients)													
1	Apr. 2005–Feb. 2009	66/M	1998	Spleen abscess	Diabetes	Polyarthralgia	+	1,280	5,120	6	0	1, Dox (60) + Strep (15); 2, Dox (60) + Rif (60) + TMP-SMZ (60); 3, Dox (180)	Polyarthralgia
2	Aug. 2005–Jan. 2009	51/M	1978	Pain in right hip	Hypertension, dyslipidemia, depression, glaucoma	Pain in right hip	−	−	1,280	271	154	Dox (90) + Rif (90)	Pain in right hip
3	June 2006–Jan. 2009	44/M	1990	Multiple multifocal motor neuropathy	Multiple multifocal motor neuropathy	Loss of right-hand strength	−	−	40	461	0	1, Dox (240) + Rif (240); 2, Dox (180) + Rif (180)	Loss of right-hand strength
4	Sept. 2006–Oct. 2007	30/M	1998	Pain in right knee	Epileptic crisis	Polyarthralgia	−	−	40	0[e]	0[e]	Dox (45) + Gent (7)	Polyarthralgia
5	Nov. 2003–Feb. 2009	35/M	1999	Sacroiliitis	Hemochromatosis, obesity, seborrheic dermatitis	Osteoarticular pain	+	20	1,280	136	35	None recommended	Polyarthralgia
6	Mar. 2007–Jan. 2009	40/F	1993	Spondylitis L5	Fibromyalgia, asthma	Arthromyalgia, lumbar pain	−	−	80	125	94	Dox (90) + Rif (90)	Osteomuscular pain
7	Dec. 2006–Sept. 2008	64/M	2000	Spondylitis	None	None	+	20	640	96	0	None recommended	Asymptomatic
8	July 2003–Dec. 2008	60/M	2000	Hepatic abscess	None	None	−	−	640	72	359	None recommended	Asymptomatic
Group B (nonfocal-disease patients with subjective complaints)													
9	Mar. 2003–Nov. 2008	44/M	1990	None	CFS, hepatitis C	Osteomuscular pain	−	−	−	178	132	None recommended	Osteomuscular pain
10	Feb. 2003–Jan. 2008	57/M	1997	None	Prostatitis	Polyarthralgia	−	−	1,280	6	0	1, Dox (60); 2, Dox (60); 3, Dox (60); 4, Dox (60)	Polyarthralgia
11	Jan. 2006–Feb. 2009	49/F	2006	None	CFS, DM II	Polyarthralgia, asthenia	+	80	320	3,755	0	1, Dox (60); 2, Dox (45) + Strep (14); 3, TMP-SMZ (90); 4, Dox (90)	Polyarthralgia
12	Dec. 2002–Feb. 2009	54/M	2002	None	Schizophrenia	Pain in right shoulder	+	40	20,480	0	797	None recommended	Asymptomatic
13	Apr. 2003–Feb. 2009	42/F	1994	None	CFS	Polyarthralgia, fatigue, asthenia	−	40	40	112	0	Dox (180)	Osteomuscular pain
14	June 2006–Jan. 2009	36/F	1996	None	None	Polyarthralgia	+	80	2,560	0	182	1, Dox + Rif (interrupted); 2, TMP-SMZ (90); 3, Dox (90)	Polyarthralgia
15	July 2006–Oct. 2008	35/M	1997	None	Hyperlipidemia	Polyarthralgia	−	−	−	32	366	Dox (120) + Rif (120)	Polyarthralgia
16	Oct. 2007–Jan. 2009	37/M	1991	None	CFS	Polyarthralgia	−	−	−	0	121	None recommended	Polyarthralgia
17	Feb. 2008–Jan. 2009	56/M	1975	None	CFS, hypothyroidism, hypertension	Polyarthralgia	−	40	2,560	25	0	Rif (interrupted)	Polyarthralgia

[a] M, male; F, female.
[b] DM II, diabetes mellitus type II.
[c] RB, rose bengal; STA, standard tube agglutination; +, positive; −, negative. Standard tube agglutination and Coombs test results are expressed as reciprocal serology titers. Q-PCR results are expressed as copies of *B. melitensis* DNA per milliliter of sample.
[d] Dox, doxycycline (100 mg/12 h); Strep, streptomycin (1 g/24 h); Rif, rifampin (900 mg/24 h); Gent, gentamicin (240 mg/24 h); TMP-SMZ, cotrimoxazole (trimethoprim [TMP], 160 mg/12 h; sulfamethoxazole [SMZ], 800 mg/12 h). Numbers followed by commas indicate the first, second, third, or fourth course of treatment.
[e] Upon entry into the study, the SF sample was positive by the Q-PCR assay, with 175 copies/ml.

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TABLE 2. Serological results for the three groups of patients

Group[a] (*n*)	Wright test		Coombs test		No. positive by the rose bengal test/ total (%)
	No. with a score of ≥1:160/total (%)	Range[b]	No. with a score of ≥1:320/total (%)	Range	
A (8)	6/90 (7)	Neg–1:1,280	45/90 (50)	Neg–1:5,120	19/90 (21)
B (9)	17/122 (14)	Neg–1:160	62/122 (51)	Neg–1:40,960	34/122 (28)
C (18)	0/34 (0)	Neg–1:40	5/34 (15)	Neg–1:640	0/34 (0)

[a] Group A, focal-disease patients; group B, nonfocal-disease patients; group C, asymptomatic subjects.
[b] Neg, negative.

either alone or in combination with rifampin (rifampicin) (900 mg/24 h), gentamicin (240 mg/24 h), streptomycin (1 g/24 h), or cotrimoxazole (trimethoprim, 160 mg/12 h; sulfamethoxazole, 800 mg/12 h). The duration of therapy ranged from 45 to 495 days (mean, 191 days). Five patients experienced a transitory resolution of symptoms, and two showed long-term improvement. The remaining four patients experienced no beneficial effects of treatment, including one patient who interrupted treatment because of adverse effects. After the completion of antibiotic treatment, follow-up for these patients ranged from 87 to 1,050 days (mean, 472 days).

We analyzed the bacterial DNA loads from diagnosis to beyond the termination of therapy for the 10 chronic brucellosis patients who completed treatment. The changes in bacterial DNA loads for these patients are shown in Fig. 1.

The mean bacterial loads prior to antibiotic therapy were 534 ± 1,149 copies/ml (10/37 samples positive) and 479 ± 506 copies/ml (6/37 samples positive) for the whole-blood and serum samples, respectively ($P > 0.05$). During treatment, the mean bacterial loads decreased to 343 ± 486 copies/ml (10/42 samples positive) in whole-blood samples and slightly increased to 480 ± 654 copies/ml (2/42 samples positive) in serum samples, without reaching statistical significance ($P > 0.05$). Upon the completion of treatment, the bacterial DNA loads decreased slightly in whole-blood samples, to 147 ± 211 copies/ml (6/58 samples positive), and increased considerably in serum samples, to 917 ± 1,282 copies/ml (8/58 samples positive), in six (60%) patients (two patients with focal complaints and four nonfocal-disease patients).

DISCUSSION

In the current study, we evaluated the usefulness of Q-PCR in the diagnosis and follow-up of chronic brucellosis patients. We studied a cohort of 35 subjects with a history of brucellosis diagnosed between 2 and 33 years previously. The most significant characteristic of infection was the long-term persistence of *B. melitensis* DNA in nonfocal-disease patients complaining of nonspecific symptoms undiagnosable by classical methods. This observation is very important insofar as it offers hope to a not insignificant group of patients who wander from physician to physician, remaining undiagnosed even though their symptoms (weakness, easy fatigability, anxiety, nervousness, and vague aches) persist. In such situations, Q-PCR could help physicians to diagnose chronic infection. Since other intracellular bacteria, such as *Mycoplasma* spp., have been linked to a number of syndromes of as yet unknown etiology, which include fibromyalgia, CFS, and sarcoidosis (4, 6, 14), it will be of interest to investigate the potential role of *Brucella* sp. infection in such syndromes.

With respect to the specificity of Q-PCR, we did not detect bacterial DNA in either whole-blood or serum samples from healthy donors who had no history of brucellosis. However, we were able to detect and measure levels of *B. melitensis* DNA in asymptomatic subjects with a history of brucellosis, albeit in a smaller proportion of patients than that in the group of symptomatic chronic patients. The clinical significance of the persistence of bacterial DNA in asymptomatic patients for long periods after antibiotic treatment is still unclear. We do not know whether this indicates that these patients could develop symptoms of brucellosis in the future. The persistence of bacterial DNA after the completion of antimicrobial treatment has also been reported for patients with other infectious diseases, such as tuberculosis, leptospirosis, and Lyme disease (8, 11, 18, 23, 25). Asymptomatic former brucellosis patients with bacterial DNA have not been adequately described in the literature, and it seems appropriate to perform follow-up studies of these patients, who probably require special vigilance in the posttreatment phase.

The detection of bacterial DNA despite negative blood cultures in symptomatic patients without focal complications suggests that they might remain infected with nondividing but

TABLE 3. Results of Q-PCR positivity and *B. melitensis* DNA loads in blood and serum samples over time for the three groups of patients

Patient group[a] (*n*)	No. of positive Q-PCR samples/total samples analyzed (*B. melitensis* DNA load[b]) at the following point:					
	Baseline		Treatment period		Posttreatment follow-up	
	Blood	Serum	Blood	Serum	Blood	Serum
A (8)	12/39 (244 ± 253)	7/38 (432 ± 505)	9/27 (316 ± 479)	4/26 (650 ± 767)	1/29 (15)	2/29 (2,516 ± 2,109)
B (9)	14/67 (515 ± 1,524)	12/65 (243 ± 222)	1/18 (599)	2/18 (142 ± 180)	7/33 (128 ± 199)	6/33 (384 ± 218)
C (18)	5/36 (171 ± 136)	2/36 (53 ± 11)				

[a] Group A, focal-disease patients; group B, nonfocal-disease patients; group C, asymptomatic subjects.
[b] Expressed as mean copies per milliliter ± SD.

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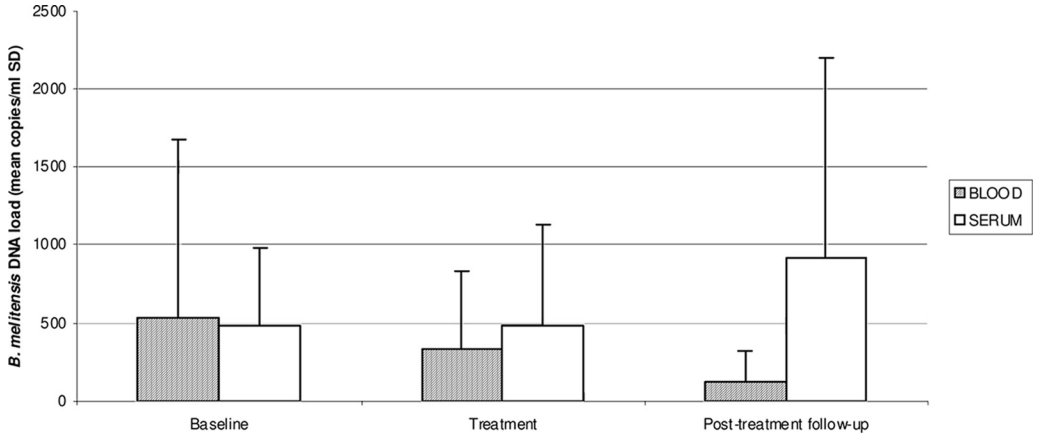

FIG. 1. Changes in mean levels of *Brucella melitensis* DNA (mean copies per milliliter ± SD) over time in treated patients (five focal-disease patients and five nonfocal-disease patients).

nonetheless live, infectious bacteria that persist in a latent form under the control of the host's immune system. Some authors (8, 11, 18) have suggested that DNA from dead microbes is rapidly cleared from the blood, presumably by nucleolytic activity present in tissues and body fluids. In an experimental model, DNA from heat-killed *Borrelia burgdorferi* was injected into the skin of an uninfected dog. PCR analysis detected *B. burgdorferi* DNA for a maximum of 3 weeks after injection, implying that during natural, acute infection, the DNA of killed microorganisms is removed quickly and completely within a few days (23). These findings could corroborate our theory that the *B. melitensis* DNA detected at least 2 years following initial brucellosis infection is derived from live bacteria with infectious, symptom-causing potential.

Since we were able to detect *B. melitensis* DNA after therapy in 80% of patients, it seems that the duration, dose, and combination of current antibiotic treatments are not effective in eradicating the bacteria. It is not clear whether therapy of longer duration or new treatments with different combinations of antibiotics would result in better outcomes. Prolonged evaluation of patients after antibiotic treatment is needed in order to determine whether the decrease in the bacterial DNA load after treatment is transient or definitive, resulting in total eradication of the bacteria.

With respect to the intermittent detection of bacterial DNA, it seems possible that in chronic brucellosis patients, the concentrations of *B. melitensis* DNA in body fluids, such as blood or serum, may be transient or too low to allow consistent detection. Furthermore, it has been reported that nucleic acids are probably released from bacteria into the circulation as breakdown products (26), which might hinder amplification by PCR. Thus, a negative result in a Q-PCR assay may not exclude the disease. Some authors have suggested testing several replicates of the purified DNA from clinical specimens in parallel to increase the probability of a positive result (1). However, in tissue samples where bacteria are concentrated, such as the bone marrow or SF from focal-disease patients, it was easier to detect *B. melitensis* DNA, as seen in the sample of SF from the young man with right-knee pain and in the bone marrow of a woman with spondylitis (16).

In summary, we found that the proportion of individuals with *B. melitensis* DNA was significantly higher for symptomatic nonfocal-disease patients than for asymptomatic subjects. Therefore, Q-PCR appears to be a useful method for identifying chronic brucellosis patients, especially those symptomatic nonfocal-disease patients for whom the classical methods of diagnosis fail.

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