Administration of a Triple versus a Standard Double Antimicrobial Regimen for Human Brucellosis More Efficiently Eliminates Bacterial DNA Load

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The effects of doxycycline-streptomycin-rifampin versus a standard doxycycline-streptomycin regimen on residual *Brucella* DNA were compared in 36 acute brucellosis patients. At admission, all patients given triple ($n = 22$) and double ($n = 14$) regimens had detectable *Brucella* DNA with similar mean loads ($P = 0.982$). At follow-up, 14 to 20 months postpresentation, significantly more patients receiving triple than double regimens had undetectable *Brucella* DNA ($P = 0.026$). The doxycycline-streptomycin-rifampin regimen eliminates *Brucella* DNA more efficiently than doxycycline-streptomycin, which may result in superior long-term clearance of *Brucella*.

Brucellosis is a zoonotic infection that is transmitted from animals to humans by direct or indirect contact with infected animals or their products, especially unpasteurized milk and cheese (1). The disease is endemic in several large geographic regions, such as South America, Middle Eastern countries, and the Mediterranean basin, including Greece (2, 3). In Greece, the mean brucellosis notification rate remains high (1.8 cases/100,000 population) based on data of the period 2005 to 2011; especially, during this period the highest rates were documented in the region of Thessaly (8.0 to 12.0 cases/100,000 population) (3, 4, 5).

Brucella may evade the immune system (6) and often produces a relapsing or chronic disease presenting with a broad spectrum of clinical manifestations and complications (1). The evasion of the human immune system implies that a prolonged therapeutic regimen with antibiotic combinations is needed to avoid treatment failure and relapses, serious complications, or residual damage from focal disease.

The complex, and usually prolonged, disease evolution limits the diagnostic applicability of conventional diagnostic tests, such as blood cultures and microbiological eradication indexes (2). Furthermore, serological tests, although useful for diagnosis, are of limited clinical applicability in predicting the outcome, since antibody response may remain detectable for a protracted period (7). These diagnostic limitations prompted the application of DNA-based approaches as more appropriate methods to evaluate the course of human brucellosis (7–10). In these studies, although PCR was found to be a valuable tool in the initial diagnosis, it remained questionable whether the detection of *Brucella* DNA in clinical samples drawn at posttreatment follow-up demands long-term antibiotic treatment or not. In a previous study, using a quantitative real-time PCR assay, we have shown that bacterial DNA loads were constantly decreasing after the end of double treatment but remained detectable in a significant number of patients for several months or years after clinical cure, without clinical symptoms (8).

The available evidence clearly indicates that the selection of appropriate treatment, which should be able to eliminate *Brucella* DNA, is still questionable and deserves further study. In that re-

spect, to investigate the most efficient combination treatment by means of *Brucella* DNA long-term eradication, we compared a triple and a standard double antibiotic scheme, by applying the above molecular protocol at two time points: at the patients' diagnosis, prior to the treatment administration, and at a follow-up visit.

Patients. The study was conducted between 2007 and 2010 in the outpatient clinic of the General Hospital of Trikala, serving the rural regional unit of Trikala, Thessaly, with approximately 200,000 inhabitants. The study included 36 prospectively recruited ambulatory patients presenting with clinical symptoms and signs indicative of acute brucellosis but no known history of brucellosis. In all cases, the presumptive clinical diagnosis of brucellosis was confirmed by positive serological assays (Rose Bengal test [RBT] and/or Wright test) and real-time PCR in peripheral whole-blood specimens. A second peripheral serum and whole-blood specimen was obtained per patient at a follow-up visit to the clinic, 14 to 20 months after the initial presentation, for serological and molecular testing.

Two treatment regimens were administered: the standard regimen of doxycycline (100 mg twice daily orally for 6 weeks) plus streptomycin (15 mg/kg of body weight daily intramuscularly for 2 weeks) was given to 14 patients (11, 12), while 22 patients presenting with more severe symptoms (such as orchiepididymitis and central nervous system disorders) or osteoarticular complications received the above scheme plus 600 mg rifampin every morning for 6 weeks (13). The study was approved by the hospital ethics committee.

Laboratory testing. Serological tests were performed for all

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patients at the initial examination and at the follow-up visit, including Rose Bengal plate (RBP) agglutination and Wright seroagglutination tests, as described previously (14).

The molecular technique was also applied in all patients; DNA isolation from whole-blood specimens and hybridization probe-based quantitative real-time PCR assay were performed as previously described (9). A LightCycler instrument (Roche Molecular Diagnostics, Mannheim, Germany), was used, with primers amplifying a 207-bp sequence of the gene encoding the BCSP31 protein, specific for the *Brucella* genus (15). Following amplification, melting curve analysis was performed to verify the specificity of PCR products. A standard curve, comprising 10-fold dilutions of *Brucella* BCSP31 DNA from 10^1 to 10^7 target equivalents, allowed quantification of unknown samples. In the provided standard row (TIB Molbiol, Berlin, Germany), the lowest concentration (10 copies) was amplified in 35 to 36 cycles and 100, 10,000, and 1,000,000 copies were amplified after 32 to 33, 25 to 26, and 17 to 18 cycles, respectively (crossing-point-calculated “second derivative maximum”). With the “fit points” method, the crossing point values were 32 to 33, 28 to 29, 22 to 23, and 15 to 16, respectively. The detection limit of the method was determined to be 10 copies/5 μ l DNA extract by spiking healthy volunteers' blood with serial dilutions of *Brucella melitensis* DNA. It should be noted that DNA was extracted from whole blood (200 μ l) with the QIAamp DNA blood minikit (Qiagen, USA) according to the manufacturer's instructions, yielding 20 μ l extract, and thus, the 5 μ l of DNA template that was applied to each real-time PCR tube was extracted from 50 μ l of patient whole blood.

Statistical analysis. Categorical variables were compared between groups using the chi-square test. Continuous variables were compared using an independent-sample *t* test. A stepwise logistic regression model was applied to identify whether age and gender played a role as confounding factors in the association between bacterial load and treatment scheme. A *P* value of <0.05 was considered statistically significant. Statistical analysis was performed with SPSS 13.0 (SPSS, Inc., Chicago, IL).

Demographic and clinical characteristics of the patient population are presented in Table 1. Twenty-one of all patients participating in the study (58%) were male, 15 (42%) were female, and their mean age was 56 years (range, 16 to 84 years). The clinical symptoms in all patients included fever, malaise, and arthralgias. Furthermore, among the 22 patients treated with the triple regimen, 18 (81.8%) presented with osteoarticular complications (index number of patients 1 to 6, 8 to 13, and 16 to 21), 8 (36.4%) presented with hepatosplenomegaly (patients 1 to 3, 8, 9, and 12 to 14), 3 (13.6%) presented with orchiepididymitis (patients 4, 14, and 15), and 2 (9.1%) presented with central nervous system disorders (patients 7 and 22) (Tables 1 and 2). In the group of patients treated with the double regimen, no such complications were presented. All patients had positive histories of milking, production, and consumption of fresh (unpasteurized) cheese and/or stock farming.

Table 2 presents the pre- and posttreatment microbiological characteristics of patients with brucellosis. At the time of diagnosis, the RBP agglutination test was positive in all 36 patients, the Wright seroagglutination test titers were within the diagnostic range (titers of ≥ 160) in 32 (89%) cases, and all patients were positive by the real-time PCR assay.

The mean load ($\pm$ standard deviation [SD]) at diagnosis was overall $1,948 \pm 1,037$ copies/5 μ l DNA extract (range, 530 to 4,321

TABLE 1 Demographic and clinical characteristics of patients with brucellosis

Patient characteristic	Value
Triple regimen (<i>n</i> = 22)	
Demographic	
No. (%) of males	13 (59)
No. (%) of females	9 (41)
Mean age in yr (range)	55 (16–82)
Clinical	
No. (%) of patients with fever	22 (100)
No. (%) of patients with constitutive symptoms (malaise, arthralgias)	22 (100)
No. (%) of patients with osteoarticular complications	18 (81)
No. (%) of patients with hepato-/splenomegaly	8 (36)
No. (%) of patients with orchiepididymitis	3 (14)
No. (%) of patients with central nervous system disorders	2 (9)
Standard regimen (<i>n</i> = 14)	
Demographic	
No. (%) of males	8 (57)
No. (%) of females	6 (43)
Mean age in yr (range)	67 (27–84)
Clinical	
No. (%) of patients with fever	14 (100)
No. (%) of patients with constitutive symptoms (malaise, arthralgias)	14 (100)

copies/5 μ l extract); the mean load of those treated with the triple regimen was $1,939 \pm 1051$ copies/5 μ l extract, similar to that of patients given the standard regimen ($1,958 \pm 958$ copies/5 μ l extract, *P* = 0.982). At the second visit, 19/22 (86.4%) patients treated with the triple scheme became negative (mean time [$\pm$ SD], 17.6 ± 2.7 months; range, 14 to 20 months), while only 7/14 (50%) patients treated with double therapy became negative (mean time [$\pm$ SD], 16.6 ± 3 months; range, 12 to 24 months) (*P* = 0.026). At the follow-up visit, serological tests (either RBT or seroagglutination test) were at least weakly positive in 14/22 patients on the triple regimen compared with 11/14 patients on the standard regimen (*P* = 0.47). Demographic parameters such as gender and age did not influence bacterial loads considerably (*P* = 0.842 and *P* = 0.146, respectively). Among 10 patients who continued to have positive real time-PCR results posttreatment, only three patients experienced symptoms indicative of active disease, as defined by reappearance of signs and symptoms of brucellosis infection (two of them received triple therapy [patients 13 and 22] and one received double treatment [patient 36]), while the rest remained asymptomatic.

Even though effective antibiotics for brucellosis are available, the problem of treating this disease has not been completely solved, and the optimal treatment regimen is debatable (16). The present study was designed in order to compare the impacts of double and triple regimens on the *Brucella* DNA long-term eradication. Patients were divided into two groups, in terms of the occurrence of uncomplicated or complicated focal disease. According to the widely accepted Ioannina Recommendations (12), the optimal treatment of acute uncomplicated brucellosis should be based on the 6-week regimen of doxycycline combined either with streptomycin for 2 to 3 weeks or with rifampin for 6 weeks. In addition, although the available evidence for the application of therapeutic schemes using more than two antibiotics is limited,

TABLE 2 Pre- and posttreatment microbiological characteristics of patients with brucellosis

Treatment group and patient no.	Pretreatment result			Posttreatment result		
	Wright test ^a	RBT	PCR (no. of copies/5 μ l DNA extract)	Wright test ^a	RBT	PCR (no. of copies/5 μ l DNA extract)
Triple regimen						
1	1/160	+	2,950	—	—	Nondetectable
2	1/320	+	4,100	—	—	Nondetectable
3	1/640	+	2,400	—	—	Nondetectable
4	—	+	724	—	—	Nondetectable
5	1/160	+	562	1/5,120	+	Nondetectable
6	1/320	+	621	—	—	Nondetectable
7	1/320	+	1,235	—	—	Nondetectable
8	1/2,560	+	3,402	—	—	Nondetectable
9	1/2,560	+	1,209	—	—	Nondetectable
10	1/2,560	+	2,150	—	—	Nondetectable
11	1/2,560	+	4,321	1/40	—	Nondetectable
12	1/320	+	834	1/320	+	125
13	1/320	+	1,840	1/2,560	+	1,950
14	1/1,280	+	2,150	1/40	—	Nondetectable
15	1/640	+	2,332	1/80	—	Nondetectable
16	1/160	+	1,930	1/40	—	Nondetectable
17	1/640	+	2,580	—	—	Nondetectable
18	1/160	+	1,650	—	—	Nondetectable
19	1/320	+	3,165	—	—	Nondetectable
20	1/1,280	+	2,203	—	—	Nondetectable
21	1/2,560	+	1,970	1/80	—	Nondetectable
22	1/320	+	1,850	1/5,120	+	2,950
% positive results				40.9	18.2	13.6
Standard regimen						
23	1/1,280	+	1,440	—	—	98
24	—	+	735	—	—	Nondetectable
25	—	+	873	—	—	85
26	1/160	+	745	—	—	137
27	1/2,560	+	3,585	—	—	120
28	1/640	+	2,500	1/80	—	130
29	1/320	+	530	—	—	Nondetectable
30	1/320	+	650	—	—	Nondetectable
31	1/640	+	1,770	—	—	Nondetectable
32	1/320	+	3,200	1/40	—	Nondetectable
33	1/1,280	+	2,840	—	—	65
34	1/640	+	1,750	—	—	Nondetectable
35	1/640	+	1,075	—	—	Nondetectable
36	1/640	+	2,250	1/5120	+	3,520
% positive results				21.4	7.1	50

^a Values of Wright test results are dilutions.

triple combinations including doxycycline, rifampin, and an aminoglycoside have proved to be more effective than only doxycycline plus an aminoglycoside in patients suffering from acute complicated disease, especially with osteoarticular complications (13, 17, 18).

Based on the above-described studies, the group with the complicated brucellosis was administered the triple drug regimen and the group with the uncomplicated disease was given the double regimen. The two treatment groups, apart from their clinical manifestations, did not differ in demographic or other characteristics that could have interfered with the statistical assessment. Moreover, despite the more severe clinical manifestations of the group administered the triple drug regimen, the eradication of microbial load presented in the follow-up was statistically higher than that in the other group, indicating a strong therapeutic im-

pact of the triple drug regimen on the mean *Brucella* microbial load, even in complicated forms of brucellosis, which are difficult to treat.

Since *Brucella* is an intracellular pathogen, microbiological eradication cannot be evaluated definitively, and thus, therapeutic efficacy is based purely on clinical grounds: absence of symptoms and signs of residual or relapsing disease (1). Furthermore, serological tests are useful in diagnosis, but the antibodies resolve slowly posttreatment, and levels of antibodies are also usually inadequate in predicting the clinical outcome. This applies even to the more sophisticated techniques such as enzyme-linked immunosorbent assay (ELISA), which has been understudied (7). The above-mentioned limitations of the phenotypic methods overall indicated that molecular biology-based estimations of the *Brucella* blood DNA load would provide valuable data for the design of

appropriate treatment regimens and prompted the present study.

In the last decade, it was generally believed that clinical judgment would be facilitated by the implementation of molecular methods. Real-time PCR is considered a powerful tool for the detection and quantification of bacterial load in the initial diagnosis of symptomatic nonfocal disease in patients, for whom classical microbiological methods fail (19), as well as for differentiation between active and past *Brucella* infections (20). However, using this technique, *Brucella* DNA was detected in the majority of brucellosis patients throughout treatment and follow-up, despite appropriate antibiotic therapy and apparent clinical recovery (8). In the latter study, no data could be extracted about whether a specific therapeutic regimen was related to the long-term presence or absence of detectable DNA, because the majority of the patients were treated with the same regimen. For this reason, in the present study we administered to 22 patients expressing more-serious symptoms a triple scheme and to the remaining 14 patients a double regimen; significantly more of the former patients than of the latter eliminated their bacterial load in their follow-up sample. In a similar Spanish study, numerous regimens were used, but the small sample for each particular regimen did not allow for firm conclusions to be drawn (9).

Although detectable *Brucella* DNA does not prove active infection, the persistence of DNA raises the possibility of latent infection inside the cells. It has been shown that *Brucella* species can survive inside macrophage cells, eluding host immune response and impeding effective antibiotic action (1). Nevertheless, four patients in a Spanish study who had negative results at the finalization of treatment relapsed, suggesting that although bacterial DNA was not present in blood, *Brucella* organisms may have been surviving intracellularly (9). In that respect, a negative *Brucella* DNA result probably rules out active brucellosis infection but does not exclude the possibility of eventual relapse. Nevertheless, it should be noted that our patients with nondetectable *Brucella* DNA load at the follow-up remained clinically healthy at least 20 months posttherapy (data not shown).

It could be rationally anticipated that the absence (or low/undetectable levels) of *Brucella* DNA would be more favorable on a long-term basis than persistent detectable DNA loads. However, the small number of relapses, which did not allow statistical evaluation, precluded a concurrent evaluation of the disease's clinical outcome in the individual study patients. Future larger-scale studies are needed to monitor the clinical, serological, and molecular outcomes of treated brucellosis patients, in order to determine optimal schemes.

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